

# *Regulation of Epithelial Tissue by Hedgehog-like Polypeptides, and Formulations and Uses Related Thereto*

## *Reference to Related Applications*

5 This application is a continuation-in-part of USSN 08/955,552, filed October 20, 1997, the specification of which is incorporated by reference herein.

## *Background of the Invention*

10 Pattern formation is the activity by which embryonic cells form ordered spatial arrangements of differentiated tissues. The physical complexity of higher organisms arises during embryogenesis through the interplay of cell-intrinsic lineage and cell-extrinsic signaling. Inductive interactions are essential to embryonic patterning in vertebrate development from the earliest establishment of the body plan, to the patterning of the organ systems, to the generation of diverse cell types during tissue differentiation (Davidson, E., (1990) *Development* 108: 365-389; Gurdon, J. B., (1992) *Cell* 68: 185-199; Jessell, T. M. et al., (1992) *Cell* 68: 257-270). The effects of developmental cell interactions are varied. Typically, responding cells are diverted from one route of cell differentiation to another by inducing cells that differ from both the uninduced and induced states of the responding cells (inductions). Sometimes cells induce their neighbors to differentiate like themselves (homoio-genetic induction); in other cases a cell inhibits its neighbors from differentiating like itself. Cell interactions in early development may be sequential, such that an initial induction between two cell types leads to a progressive amplification of diversity. Moreover, inductive interactions occur not only in embryos, but in adult cells as well, and can act to establish and maintain morphogenetic patterns as well as induce differentiation (J.B. Gurdon (1992) *Cell* 68:185-199).

25 Members of the *Hedgehog* family of signaling molecules mediate many important short- and long-range patterning processes during invertebrate and vertebrate development. In the fly a single *hedgehog* gene regulates segmental and imaginal disc patterning. In contrast, in vertebrates a *hedgehog* gene family is involved in the control of left-right asymmetry, polarity in the CNS, somites and limb, organogenesis, chondrogenesis and spermatogenesis.

30 The first *hedgehog* gene was identified by a genetic screen in the fruitfly *Drosophila melanogaster* (Nüsslein-Volhard, C. and Wieschaus, E. (1980) *Nature* 287, 795-801). This screen identified a number of mutations affecting embryonic and larval development. In 1992 and 1993, the molecular nature of the *Drosophila hedgehog (hh)* gene was reported (C.F., Lee et

al. (1992) *Cell* 71, 33-50), and since then, several *hedgehog* homologues have been isolated from various vertebrate species. While only one *hedgehog* gene has been found in *Drosophila* and other invertebrates, multiple *Hedgehog* genes are present in vertebrates.

5 The various Hedgehog proteins consist of a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. *et al.* (1992) *Cell* 71:33-50; Tabata, T. *et al.* (1992) *Genes Dev.* 2635-2645; Chang, D.E. *et al.* (1994) *Development* 120:3339-3353), Hedgehog precursor proteins undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) *Science* 266:1528-1537; Porter *et al.* (1995) *Nature* 10 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) *supra*; Tabata *et al.* (1992) *supra*; Chang *et al.* (1994) *supra*; Lee *et al.* (1994) *supra*; Bumcrot, D.A., *et al.* (1995) *Mol. Cell. Biol.* 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) *Curr. Biol.* 5:944-955; Lai, C.J. *et al.* (1995) *Development* 121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in 15 which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra*; Mart', E. *et al.* (1995) *Development* 121:2537-2547; Roelink, H. *et al.* (1995) *Cell* 81:445-455). Interestingly, cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of HH encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) *Cell* 86, 21-34). 20 Biochemical studies have shown that the autoproteolytic cleavage of the HH precursor protein proceeds through an internal thioester intermediate which subsequently is cleaved in a nucleophilic substitution. It is likely that the nucleophile is a small lipophilic molecule which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), 25 tethering it to the cell surface. The biological implications are profound. As a result of the tethering, a high local concentration of N-terminal Hedgehog peptide is generated on the surface of the Hedgehog producing cells. It is this N-terminal peptide which is both necessary and sufficient for short and long range Hedgehog signaling activities in *Drosophila* and vertebrates (Porter *et al.* (1995) *supra*; Ekker *et al.* (1995) *supra*; Lai *et al.* (1995) *supra*; Roelink, H. *et al.* 30 (1995) *Cell* 81:445-455; Porter *et al.* (1996) *supra*; Fietz, M.J. *et al.* (1995) *Curr. Biol.* 5:643-651; Fan, C.-M. *et al.* (1995) *Cell* 81:457-465; Mart', E., *et al.* (1995) *Nature* 375:322-325; Lopez-Martinez *et al.* (1995) *Curr. Biol.* 5:791-795; Ekker, S.C. *et al.* (1995) *Development* 121:2337-2347; Forbes, A.J. *et al.* (1996) *Development* 122:1125-1135).

HH has been implicated in short- and long range patterning processes at various sites 35 during *Drosophila* development. In the establishment of segment polarity in early embryos, it has

short range effects which appear to be directly mediated, while in the patterning of the imaginal discs, it induces long range effects via the induction of secondary signals.

In vertebrates, several *hedgehog* genes have been cloned in the past few years (see Table 1). Of these genes, *Shh* has received most of the experimental attention, as it is expressed in different organizing centers which are the sources of signals that pattern neighbouring tissues. Recent evidence indicates that *Shh* is involved in these interactions.

The interaction of a hedgehog protein with one of its cognate receptor, *patched*, sets in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or translation of a gene. Transcriptional targets of *hedgehog* signaling are the *patched* gene itself (Hidalgo and Ingham, 1990 *Development* 110, 291-301; Marigo et al., 1996 ) and the vertebrate homologs of the drosophila cubitus interruptus (Ci) gene, the *GLI* genes (Hui et al. (1994) *Dev Biol* 162:402-413). *Patched* gene expression has been shown to be induced in cells of the limb bud and the neural plate that are responsive to *Shh*. (Marigo et al. (1996) *Development* 122:1225-1233). The *GLI* genes encode putative transcription factors having zinc finger DNA binding domains (Orenic et al. (1990) *Genes & Dev* 4:1053-1067; Kinzler et al. (1990) *Mol Cell Biol* 10:634-642). Transcription of the *GLI* gene has been reported to be upregulated in response to *hedgehog* in limb buds, while transcription of the *GLI3* gene is downregulated in response to *hedgehog* induction (Marigo et al. (1996) *Development* 122:1225-1233). Moreover, it has been demonstrated that elevated levels of Ci are sufficient to activate *patched* (*ptc*) and other *hedgehog* target genes, even in the absence of *hedgehog* activity.

### *Summary of the Invention*

One aspect of the present application relates to a method for modulating the growth state of an epithelial cell by ectopically contacting the epithelial cell, *in vitro* or *in vivo*, with a hedgehog therapeutic or *ptc* therapeutic in an amount effective to alter the rate (promote or inhibit) of proliferation of the epithelial cell, e.g., relative to the absence of administration of the hedgehog therapeutic or *ptc* therapeutic. The subject method can be used, for example, to modulate the growth state of an epithelial tissue, such as for inducing the formation of skin or other cutaneous tissue, or for inducing growth of hair.

Wherein the subject method is carried out using a *hedgehog* therapeutic, the *hedgehog* therapeutic preferably a polypeptide including a *hedgehog* portion comprising at least a bioactive extracellular portion of a *hedgehog* protein, e.g., the *hedgehog* portion includes at least 50, 100 or 150 (contiguous) amino acid residues of an N-terminal half of a *hedgehog* protein. In preferred

embodiments, the *hedgehog* portion includes at least a portion of the *hedgehog* protein corresponding to a 19kd fragment of the extracellular domain of a *hedgehog* protein.

In certain preferred embodiments, the *hedgehog* portion has an amino acid sequence at least 60, 75, 85, or 95 percent identical with a *hedgehog* protein of any of SEQ ID Nos. 10-18 or 20, though sequences identical to those sequence listing entries are also contemplated as useful in the present method. The *hedgehog* portion can be encoded by a nucleic acid which hybridizes under stringent conditions to a nucleic acid sequence of any of SEQ ID Nos. 1-9 or 19, e.g., the *hedgehog* portion can be encoded by a vertebrate *hedgehog* gene, especially a human *hedgehog* gene.

In certain embodiments, the *hedgehog* polypeptide is modified with one or more sterol moieties, e.g., cholesterol or a derivative thereof.

In certain embodiments, the *hedgehog* polypeptide is modified with one or more fatty acid moieties, such as a fatty acid moiety selected from the group consisting of myristoyl, palmitoyl, stearoyl, and arachidoyl.

In certain embodiments, the *hedgehog* polypeptide is modified with one or more aromatic hydrocarbons, such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, or naphthacene.

In certain embodiments, the *hedgehog* polypeptide is modified one or more times with a C7 - C30 alkyl or cycloalkyl.

In other embodiments, the subject method can be carried out by administering a gene activation construct, wherein the gene activation construct is designed to recombine with a genomic *hedgehog* gene of the patient to provide a heterologous transcriptional regulatory sequence operatively linked to a coding sequence of the *hedgehog* gene.

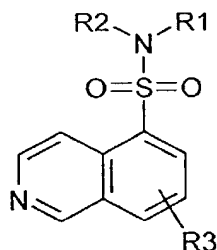
In still other embodiments, the subject method can be practiced with the administration of a gene therapy construct encoding a *hedgehog* polypeptide. For instance, the gene therapy construct can be provided in a composition selected from a group consisting of a recombinant viral particle, a liposome, and a poly-cationic nucleic acid binding agent,

In yet other embodiments, the subject method can be carried out using a ptc therapeutic. An exemplary ptc therapeutic is a small organic molecule which binds to a *patched* protein and derepresses *patched*-mediated inhibition of mitosis, e.g., a molecule which binds to *patched* and mimics *hedgehog*-mediated *patched* signal transduction, which binds to *patched* and regulates *patched*-dependent gene expression. For instance, the binding of the ptc therapeutic to *patched* may result in upregulation of *patched* and/or *gli* expression.

In a more generic sense, the *ptc* therapeutic can be a small organic molecule which interacts with epithelial cells to induce *hedgehog*-mediated *patched* signal transduction, such as by altering the localization, protein-protein binding and/or enzymatic activity of an intracellular protein involved in a *patched* signal pathway. For instance, the *ptc* therapeutic may alter the level of expression of a *hedgehog* protein, a *patched* protein or a protein involved in the intracellular signal transduction pathway of *patched*.

In certain embodiments, the *ptc* therapeutic is an antisense construct which inhibits the expression of a protein which is involved in the signal transduction pathway of *patched* and the expression of which antagonizes *hedgehog*-mediated signals. The antisense construct is preferably an oligonucleotide of about 20-30 nucleotides in length and having a GC content of at least 50 percent.

In other embodiments, the *ptc* therapeutic is an inhibitor of protein kinase A (PKA), such as a 5-isoquinolinesulfonamide. The PKA inhibitor can be a cyclic AMP analog. Exemplary PKA inhibitors include N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, KT5720, 8-bromo-cAMP, dibutyryl-cAMP and PKA Heat Stable Inhibitor isoform  $\alpha$ . Another exemplary PKA inhibitor is represented in the general formula:



wherein,

$R_1$  and  $R_2$  each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido,  $-(CH_2)_m-R_8$ ,  $-(CH_2)_m-OH$ ,  $-(CH_2)_m-O$ -lower alkyl,  $-(CH_2)_m-O$ -lower alkenyl,  $-(CH_2)_n-O$ - $(CH_2)_m-R_8$ ,  $-(CH_2)_m-SH$ ,  $-(CH_2)_m-S$ -lower alkyl,  $-(CH_2)_m-S$ -lower alkenyl,  $-(CH_2)_n-S-(CH_2)_m-R_8$ , or

$R_1$  and  $R_2$  taken together with N form a heterocycle (substituted or unsubstituted);

$R_3$  is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido,  $-(CH_2)_m-$   
5  $R_8$ ,  $-(CH_2)_m-OH$ ,  $-(CH_2)_m-O$ -lower alkyl,  $-(CH_2)_m-O$ -lower alkenyl,  $-(CH_2)_n-O-(CH_2)_m-R_8$ ,  $-(CH_2)_m-SH$ ,  $-(CH_2)_m-S$ -lower alkyl,  $-(CH_2)_m-S$ -lower alkenyl,  $-(CH_2)_n-S-(CH_2)_m-R_8$ ;

$R_8$  represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

$n$  and  $m$  are independently for each occurrence zero or an integer in the range of 1 to 6.

10 The subject method can be used to treat, e.g., a epithelial disorder, such as in the control of a wound healing process. For instance, the subject method can be used as part of such treatments as burn treatment, skin regeneration, skin grafting, pressure sore treatment, dermal ulcer treatment, post surgery scar reduction and treatment of ulcerative colitis. In the control of hair growth, the subject method can be used as part of a treatment of alopecia.

15 Yet another aspect of the present invention concerns preparations of a *hedgehog* or *ptc* therapeutic formulated for topical application to epithelial tissue, e.g., to skin. For example, such formulations may include a polypeptide comprising a hedgehog polypeptide sequence including a bioactive fragment of a *hedgehog* protein, which polypeptide is formulated for topical application to epithelial tissue.

#### 20 *Detailed Description of the Invention*

Figures 1A, B and C illustrate the induction of hair growth on mice treated with various hedgehog formulations.

#### 25 *Detailed Description of the Invention*

Normal skin epidermis is a complex epithelial tissue containing keratinocytes that are proliferating, differentiating and desquamating, and is stratified such that morphological and functional changes in the keratinocytes occur in an orderly progression. The normal epidermis is maintained in a dynamic steady state as proliferation of keratinocytes continually compensates  
30 for the loss of cells which are shed from the surface of the skin. Within the epidermis, proliferation takes place in the basal layer of keratinocytes that are attached to the underlying basement membrane, and cells undergo terminal differentiation as they migrate through the suprabasal layers, finally being shed from the tissue surface as dead, cornified squames. Three

subpopulations of basal keratinocytes have been defined by cell kinetic analysis: stem cells, transit-amplifying cells, and committed cells. Stem cells retain a high capacity for self-renewal throughout adult life and are ultimately responsible for epidermal maintenance and repair. The progeny of stem cells can either be stem cells themselves or cells known as transit-amplifying cells. Transit-amplifying cells divide a small number of times, but have a high probability of producing daughters that withdraw irreversibly from the cell cycle and are committed to differentiate terminally.

#### I. Overview

The present application is directed to the discovery that preparations of hedgehog polypeptides can be used to control the formation and/or maintenance of epithelial tissue. As described in the appended examples, *hedgehog* proteins are implicated in the proliferation of epithelial stem cells and may provide early signals that regulate the differentiation of the stem cells into epithelial tissues. In general, the method of the present invention comprises contacting an epithelial cell with an amount of a hedgehog therapeutic (defined *infra*) which produces a non-toxic response by the cell of (i) induction of epithelial tissue formation or (ii) inhibition of epithelial tissue formation, depending on the whether the hedgehog therapeutic is a sufficient hedgehog agonist or hedgehog antagonist. The subject method can be carried out on epithelial cells which may be either dispersed in culture or a part of an intact tissue or organ. Moreover, the method can be performed on cells which are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*).

In one aspect, the present invention provides pharmaceutical preparations and methods for controlling the proliferation of epithelially-derived tissue utilizing, as an active ingredient, a *hedgehog* polypeptide or a mimetic thereof. The invention also relates to methods of controlling proliferation of epithelial-derived tissue by use of the pharmaceutical preparations of the invention.

The *hedgehog* formulations of the present invention may be used as part of regimens in the treatment of disorders of, or surgical or cosmetic repair of, such epithelial tissues as skin and skin organs; corneal, lens and other ocular tissue; mucosal membranes; and periodontal epithelium. The methods and compositions disclosed herein provide for the treatment or prevention of a variety of damaged epithelial and mucosal tissues. For instance, the subject method can be used to control wound healing processes, as for example may be desirable in connection with any surgery involving epithelial tissue, such as from dermatological or periodontal surgeries. Exemplary surgical repair for which *hedgehog* therapy is a candidate

treatment include severe burn and skin regeneration, skin grafts, pressure sores, dermal ulcers, fissures, post surgery scar reduction, and ulcerative colitis.

In another aspect of the present invention, *hedgehog* preparations can be used to effect the growth of hair, as for example in the treatment of alopecia whereby hair growth is potentiated, or  
5 for example in cosmetic removal of hair (depilation) whereby hair growth is inhibited.

In certain embodiments, the subject compositions can be used to inhibit, rather than promote, growth of epithelial-derived tissue. For instance, certain of the compositions disclosed herein may be applied to the treatment or prevention of a variety hyperplastic or neoplastic conditions. The method can find application for the treatment or prophylaxis of, e.g., psoriasis;  
10 keratosis; acne; comedogenic lesions; folliculitis and pseudofolliculitis; keratoacanthoma; callosities; Darier's disease; ichthyosis; lichen planus; molluscous contagiosum; melasma; Fordyce disease; and keloids or hypertrophic scars. Certain of the formulations of the present invention may also be used as part of treatment regimens in auto-immune diseases for affecting healing of proliferative manifestations of the disorder, as for example, part of a treatment for  
15 aphthous ulcers, pemphigus such as pemphigus vulgaris, pemphigus foliaceus, pemphigus vegetans or pemphigus erythematous, epidermolysis, lupus lesions or desquamative lesions.

The subject *hedgehog* treatments are effective on both human and animal subjects afflicted with these conditions. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are  
20 dogs, cats, cattle, horses, sheep, hogs and goats.

Still another aspect of the present invention provides a method of stimulating the growth and regulating the differentiation of epithelial tissue in tissue culture.

Without wishing to be bound by any particular theory, the induction of stem cell proliferation by *hedgehog* proteins may be due at least in part to the ability of these proteins to  
25 antagonize (directly or indirectly) *patched*-mediated regulation of gene expression and other physiological effects mediated by that protein. The *patched* gene product, a cell surface protein, is understood to signal through a pathway which causes transcriptional repression of members of the Wnt and Dpp/BMP families of morphogens, proteins which impart positional information. In development of the CNS and patterning of limbs in vertebrates, the introduction of *hedgehog*  
30 relieves (derepresses) this inhibition conferred by *patched*, allowing expression of particular gene programs.

Recently, it has been reported that mutations in the human version of *patched*, a gene first identified in a fruit fly developmental pathway, cause a hereditary skin cancer and may contribute to sporadic skin cancers. See, for example, Hahn et al. (1996) *Cell* 86:841-851; and



Johnson et al. (1996) *Science* 272:1668-1671. The demonstration that nevoid basal-cell carcinoma (NBCC) results from mutations in the human *patched* gene provided an example of the roles *patched* plays in post-embryonic development. These observations have led the art to understand one activity of *patched* to be a tumor suppressor gene, which may act by inhibiting proliferative signals from *hedgehog*. Our observations set forth below reveal potential new roles for the *hedgehog/patched* pathway in maintenance of epithelial cell proliferation and differentiation. Accordingly, the present invention contemplates the use of other agents which are capable of mimicking the effect of the *hedgehog* protein on *patched* signalling, e.g., as may be identified from the drug screening assays described below.

## II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The term "hedgehog therapeutic" refers to various forms of hedgehog polypeptides, as well as peptidomimetics, which can modulate the proliferation/differentiation state of epithelial cells by, as will be clear from the context of individual examples, mimicing or potentiating (agonizing) or inhibiting (antagonizing) the effects of a naturally-occurring *hedgehog* protein. A *hedgehog* therapeutic which mimics or potentiates the activity of a wild-type hedgehog protein is a "hedgehog agonist". Conversely, a *hedgehog* therapeutic which inhibits the activity of a wild-type hedgehog protein is a "hedgehog antagonist".

In particular, the term "hedgehog polypeptide" encompasses preparations of *hedgehog* proteins and peptidyl fragments thereof, both agonist and antagonist forms as the specific context will make clear.

As used herein the term "bioactive fragment of a *hedgehog* protein" refers to a fragment of a full-length *hedgehog* polypeptide, wherein the fragment specifically agonizes or antagonizes inductive events mediated by wild-type *hedgehog* proteins. The *hedgehog* bioactive fragment preferably is a soluble extracellular portion of a *hedgehog* protein, where solubility is with reference to physiologically compatible solutions. Exemplary bioactive fragments are described in PCT publications WO 95/18856 and WO 96/17924.

The term "patched" or "ptc" refers to a family of related transmembrane proteins which have been implicated in the signal transduction induced by contacting a cell with a *hedgehog* protein. For example, the mammalian ptc family includes ptc1 and ptc2. In addition to references set out below, see also Takabatake et al. (1997) *FEBS Lett* 410:485 and GenBank AB000847 for examples of ptc2. Unless otherwise evident from the context, it will be

understood that embodiments described in the context of ptc1 (or just ptc) also refer to equivalent embodiments involving other ptc homologs like ptc2.

5 The term "ptc therapeutic" refers to agents which either (i) mimic the effect of *hedgehog* proteins on *patched* signalling, e.g., which antagonize the cell-cycle inhibitory activity of *patched*, or (ii) activate or potentiate *patched* signalling. In other embodiments, the ptc therapeutic can be a *hedgehog* antagonist. The ptc therapeutic can be, e.g., a peptide, a nucleic acid, a carbohydrate, a small organic molecule, or natural product extract (or fraction thereof).

10 A "proliferative" form of a hedgehog or ptc therapeutic is one which induces proliferation of epithelial cells, particularly epithelial stem cells. Conversely, an "antiproliferative" form of a hedgehog or ptc therapeutic is one which inhibits proliferation of an epithelial cells, preferably in a non-toxic manner, e.g., by promoting or maintaining a differentiated phenotype or otherwise promoting quiescence.

15 By way of example, though not wishing to be bound by a particular theory, proliferative hedgehog polypeptide will generally be a form of the protein which derepresses patched-mediated cell-cycle arrest, e.g., the polypeptide mimics the effect of a naturally occurring hedgehog protein effect on epithelial cells. A proliferative ptc therapeutic includes other agents which depress patched-mediated cell-cycle arrest, and may act extracellularly or intracellularly.

20 An illustrative antiproliferative ptc therapeutic agent may potentiate patched-mediated cell-cycle arrest. Such agents can be small molecules that inhibit, e.g., hedgehog binding to patched, as well as agents which stimulate and/or potentiate a signal transduction pathway of the patched protein.

25 The terms "epithelia", "epithelial" and "epithelium" refer to the cellular covering of internal and external body surfaces (cutaneous, mucous and serous), including the glands and other structures derived therefrom, e.g., corneal, esophageal, epidermal, and hair follicle epithelial cells. Other exemplary epithelial tissue includes: olfactory epithelium, which is the pseudostratified epithelium lining the olfactory region of the nasal cavity, and containing the receptors for the sense of smell; glandular epithelium, which refers to epithelium composed of secreting cells; squamous epithelium, which refers to epithelium composed of flattened plate-like cells. The term epithelium can also refer to transitional epithelium, which that characteristically  
30 found lining hollow organs that are subject to great mechanical change due to contraction and distention, e.g. tissue which represents a transition between stratified squamous and columnar epithelium.

The term "epithelialization" refers to healing by the growth of epithelial tissue over a denuded surface.

The term "skin" refers to the outer protective covering of the body, consisting of the corium and the epidermis, and is understood to include sweat and sebaceous glands, as well as hair follicle structures. Throughout the present application, the adjective "cutaneous" may be used, and should be understood to refer generally to attributes of the skin, as appropriate to the context in which they are used.

The term "epidermis" refers to the outermost and nonvascular layer of the skin, derived from the embryonic ectoderm, varying in thickness from 0.07-1.4 mm. On the palmar and plantar surfaces it comprises, from within outward, five layers: basal layer composed of columnar cells arranged perpendicularly; prickle-cell or spinous layer composed of flattened polyhedral cells with short processes or spines; granular layer composed of flattened granular cells; clear layer composed of several layers of clear, transparent cells in which the nuclei are indistinct or absent; and horny layer composed of flattened, cornified non-nucleated cells. In the epidermis of the general body surface, the clear layer is usually absent.

The "corium" or "dermis" refers to the layer of the skin deep to the epidermis, consisting of a dense bed of vascular connective tissue, and containing the nerves and terminal organs of sensation. The hair roots, and sebaceous and sweat glands are structures of the epidermis which are deeply embedded in the dermis.

The term "nail" refers to the horny cutaneous plate on the dorsal surface of the distal end of a finger or toe.

The term "epidermal gland" refers to an aggregation of cells associated with the epidermis and specialized to secrete or excrete materials not related to their ordinary metabolic needs. For example, "sebaceous glands" are holocrine glands in the corium that secrete an oily substance and sebum. The term "sweat glands" refers to glands that secrete sweat, situated in the corium or subcutaneous tissue, opening by a duct on the body surface.

The term "hair" refers to a threadlike structure, especially the specialized epidermal structure composed of keratin and developing from a papilla sunk in the corium, produced only by mammals and characteristic of that group of animals. Also, the aggregate of such hairs. A "hair follicle" refers to one of the tubular-invaginations of the epidermis enclosing the hairs, and from which the hairs grow; and "hair follicle epithelial cells" refers to epithelial cells which surround the dermal papilla in the hair follicle, e.g., stem cells, outer root sheath cells, matrix cells, and inner root sheath cells. Such cells may be normal non-malignant cells, or transformed/immortalized cells.

The term "nasal epithelial tissue" refers to nasal and olfactory epithelium.

"Excisional wounds" include tears, abrasions, cuts, punctures or lacerations in the epithelial layer of the skin and may extend into the dermal layer and even into subcutaneous fat and beyond. Excisional wounds can result from surgical procedures or from accidental penetration of the skin.

5 "Burn wounds" refer to cases where large surface areas of skin have been removed or lost from an individual due to heat and/or chemical agents.

"Dermal skin ulcers" refer to lesions on the skin caused by superficial loss of tissue, usually with inflammation. Dermal skin ulcers which can be treated by the method of the present invention include decubitus ulcers, diabetic ulcers, venous stasis ulcers and arterial ulcers.  
10 Decubitus wounds refer to chronic ulcers that result from pressure applied to areas of the skin for extended periods of time. Wounds of this type are often called bedsores or pressure sores. Venous stasis ulcers result from the stagnation of blood or other fluids from defective veins. Arterial ulcers refer to necrotic skin in the area around arteries having poor blood flow.

"Dental tissue" refers to tissue in the mouth which is similar to epithelial tissue, for example gum tissue. The method of the present invention is useful for treating periodontal disease.  
15

"Internal epithelial tissue" refers to tissue inside the body which has characteristics similar to the epidermal layer in the skin. Examples include the lining of the intestine. The method of the present invention is useful for promoting the healing of certain internal wounds, for example wounds resulting from surgery.  
20

A "wound to eye tissue" refers to severe dry eye syndrome, corneal ulcers and abrasions and ophthalmic surgical wounds.

Throughout this application, the term "proliferative skin disorder" refers to any disease/disorder of the skin marked by unwanted or aberrant proliferation of cutaneous tissue.  
25 These conditions are typically characterized by epidermal cell proliferation or incomplete cell differentiation, and include, for example, X-linked ichthyosis, psoriasis, atopic dermatitis, allergic contact dermatitis, epidermolytic hyperkeratosis, and seborrheic dermatitis. For example, epidermodysplasia is a form of faulty development of the epidermis. Another example is "epidermolysis", which refers to a loosened state of the epidermis with formation of blebs and  
30 bullae either spontaneously or at the site of trauma.

The term "carcinoma" refers to a malignant new growth made up of epithelial cells tending to infiltrate surrounding tissues and to give rise to metastases. Exemplary carcinomas include: "basal cell carcinoma", which is an epithelial tumor of the skin that, while seldom metastasizing, has potentialities for local invasion and destruction; "squamous cell carcinoma",

which refers to carcinomas arising from squamous epithelium and having cuboid cells; "carcinosarcoma", which include malignant tumors composed of carcinomatous and sarcomatous tissues; "adenocystic carcinoma", carcinoma marked by cylinders or bands of hyaline or mucinous stroma separated or surrounded by nests or cords of small epithelial cells, occurring in the mammary and salivary glands, and mucous glands of the respiratory tract; "epidermoid carcinoma", which refers to cancerous cells which tend to differentiate in the same way as those of the epidermis; i.e., they tend to form prickle cells and undergo cornification; "nasopharyngeal carcinoma", which refers to a malignant tumor arising in the epithelial lining of the space behind the nose; and "renal cell carcinoma", which pertains to carcinoma of the renal parenchyma composed of tubular cells in varying arrangements. Another carcinomatous epithelial growth is "papillomas", which refers to benign tumors derived from epithelium and having a papillomavirus as a causative agent; and "epidermoidomas", which refers to a cerebral or meningeal tumor formed by inclusion of ectodermal elements at the time of closure of the neural groove.

As used herein, the term "psoriasis" refers to a hyperproliferative skin disorder which alters the skin's regulatory mechanisms. In particular, lesions are formed which involve primary and secondary alterations in epidermal proliferation, inflammatory responses of the skin, and an expression of regulatory molecules such as lymphokines and inflammatory factors. Psoriatic skin is morphologically characterized by an increased turnover of epidermal cells, thickened epidermis, abnormal keratinization, inflammatory cell infiltrates into the dermis layer and polymorphonuclear leukocyte infiltration into the epidermis layer resulting in an increase in the basal cell cycle. Additionally, hyperkeratotic and parakeratotic cells are present.

The term "keratosis" refers to proliferative skin disorder characterized by hyperplasia of the horny layer of the epidermis. Exemplary keratotic disorders include keratosis follicularis, keratosis palmaris et plantaris, keratosis pharyngea, keratosis pilaris, and actinic keratosis.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

As used herein, "transformed cells" refers to cells which have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control.

As used herein, "immortalized cells" refers to cells which have been altered via chemical and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

A "patient" or "subject" to be treated by the subject method can mean either a human or non-human animal.

The term "cosmetic preparation" refers to a form of a pharmaceutical preparation which is formulated for topical administration.

5 An "effective amount" of, e.g., a hedgehog therapeutic, with respect to the subject method of treatment, refers to an amount of, e.g., a hedgehog polypeptide in a preparation which, when applied as part of a desired dosage regimen brings about a change in the rate of cell proliferation and/or the state of differentiation of a cell so as to produce an amount of epithelial cell proliferation according to clinically acceptable standards for the disorder to be treated or the  
10 cosmetic purpose.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

15 "Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous at that  
20 position. A percentage of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with an AR sequence of the present invention.

25 The term "corresponds to", when referring to a particular polypeptide or nucleic acid sequence is meant to indicate that the sequence of interest is identical or homologous to the reference sequence to which it is said to correspond.

30 The terms "recombinant protein", "heterologous protein" and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression construct which is in turn used to transform a host cell to produce the heterologous protein. That is, the polypeptide is expressed from a heterologous nucleic acid.

A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding a *hedgehog* polypeptide with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of *hh* protein. A chimeric protein may

present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula  $(X)_n-(hh)_m-(Y)_n$ , wherein *hh* represents all or a portion of the *hedgehog* protein, X and Y each independently represent an amino acid sequences which are not naturally found as a polypeptide chain contiguous with the *hedgehog* sequence, m is an integer greater than or equal to 1, and each occurrence of n is, independently, 0 or an integer greater than or equal to 1 (n and m are preferably no greater than 5 or 10).

10 *III. Exemplary Applications of Method and Compositions*

09037410:04550  
T05040:072360  
The subject method has wide applicability to the treatment or prophylaxis of disorders afflicting epithelial tissue, as well as in cosmetic uses. In general, the method can be characterized as including a step of administering to an animal an amount of a *ptc* or *hedgehog* therapeutic effective to alter the proliferative state of a treated epithelial tissue. The mode of administration and dosage regimens will vary depending on the epithelial tissue(s) which is to be treated. For example, topical formulations will be preferred where the treated tissue is epidermal tissue, such as dermal or mucosal tissues. Likewise, as described in further detail below, the use of a particular *ptc* or *hedgehog* therapeutic, e.g., an agonist or antagonist, will depend on whether proliferation of cells of the treated tissue is desired or intended to be prevented.

20 A method which "promotes the healing of a wound" results in the wound healing more quickly as a result of the treatment than a similar wound heals in the absence of the treatment. "Promotion of wound healing" can also mean that the method causes the proliferation and growth of, *inter alia*, keratinocytes, or that the wound heals with less scarring, less wound contraction, less collagen deposition and more superficial surface area. In certain instances, "promotion of wound healing" can also mean that certain methods of wound healing have improved success rates, (e.g. the take rates of skin grafts,) when used together with the method of the present invention.

30 Complications are a constant risk with wounds that have not fully healed and remain open. Although most wounds heal quickly without treatment, some types of wounds resist healing. Wounds which cover large surface areas also remain open for extended periods of time. In one embodiment of the present invention, the subject method can be used to accelerate the healing of wounds involving epithelial tissues, such as resulting from surgery, burns, inflammation or irritation. Certain of the *hedgehog* and *ptc* therapeutic formulations (e.g., proliferative forms) of the present invention can also be applied prophylactically, such as in the

form of a cosmetic preparation, to enhance tissue regeneration processes, e.g., of the skin, hair and/or fingernails.

Despite significant progress in reconstructive surgical techniques, scarring can be an important obstacle in regaining normal function and appearance of healed skin. This is particularly true when pathologic scarring such as keloids or hypertrophic scars of the hands or face causes functional disability or physical deformity. In the severest circumstances, such scarring may precipitate psychosocial distress and a life of economic deprivation. Wound repair includes the stages of hemostasis, inflammation, proliferation, and remodeling. The proliferative stage involves multiplication of fibroblasts and endothelial and epithelial cells. Through the use of the subject method, the rate of proliferation of epithelial cells in and proximal to the wound can be controlled in order to accelerate closure of the wound and/or minimize the formation of scar tissue.

Full and partial thickness burns are an example of a wound type which often covers large surface areas and therefore requires prolonged periods of time to heal. As a result, life-threatening complications such as infection and loss of bodily fluids often arise. In addition, healing in burns is often disorderly, resulting in scarring and disfigurement. In some cases wound contraction due to excessive collagen deposition results in reduced mobility of muscles in the vicinity of the wound. The compositions and method of the present invention can be used to accelerate the rate of healing of burns and to promote healing processes that result in more desirable cosmetic outcomes and less wound contraction and scarring.

Severe burns which cover large areas are often treated by skin autografts taken from undamaged areas of the patient's body. The subject method can also be used in conjunction with skin grafts to improve "take" rates of the graft by accelerating growth of both the grafted skin and the patient's skin that is proximal to the graft.

Dermal ulcers are yet another example of wounds that are amenable to treatment by the subject method, e.g., to cause healing of the ulcer and/or to prevent the ulcer from becoming a chronic wound. For example, one in seven individuals with diabetes develop dermal ulcers on their extremities, which are susceptible to infection. Individuals with infected diabetic ulcers often require hospitalization, intensive services, expensive antibiotics, and, in some cases, amputation. Dermal ulcers, such as those resulting from venous disease (venous stasis ulcers), excessive pressure (decubitus ulcers) and arterial ulcers also resist healing. The prior art treatments are generally limited to keeping the wound protected, free of infection and, in some cases, to restore blood flow by vascular surgery. According to the present method, the afflicted area of skin can be treated by a therapy which includes a *hedgehog* or *ptc* therapeutic which promotes epithelization of the wound, e.g., accelerates the rate of the healing of the skin ulcers.



The present treatment can also be effective as part of a therapeutic regimen for treating oral and paraoral ulcers, e.g. resulting from radiation and/or chemotherapy. Such ulcers commonly develop within days after chemotherapy or radiation therapy. These ulcers usually begin as small, painful irregularly shaped lesions usually covered by a delicate gray necrotic membrane and surrounded by inflammatory tissue. In many instances, lack of treatment results in proliferation of tissue around the periphery of the lesion on an inflammatory basis. For instance, the epithelium bordering the ulcer usually demonstrates proliferative activity, resulting in loss of continuity of surface epithelium. These lesions, because of their size and loss of epithelial integrity, lend the body to potential secondary infection. Routine ingestion of food and water becomes a very painful event and, if the ulcers proliferate throughout the alimentary canal, diarrhea usually is evident with all its complicating factors. According to the present invention, a treatment for such ulcers which includes application of an hedgehog therapeutic can reduce the abnormal proliferation and differentiation of the affected epithelium, helping to reduce the severity of subsequent inflammatory events.

In another exemplary embodiment, the subject method is provided for treating or preventing gastrointestinal diseases. Briefly, a wide variety of diseases are associated with disruption of the gastrointestinal epithelium or villi, including chemotherapy- and radiation-therapy-induced enteritis (i.e. gut toxicity) and mucositis, peptic ulcer disease, gastroenteritis and colitis, villus atrophic disorders, and the like. For example, chemotherapeutic agents and radiation therapy used in bone marrow transplantation and cancer therapy affect rapidly proliferating cells in both the hematopoietic tissues and small intestine, leading to severe and often dose-limiting toxicities. Damage to the small intestine mucosal barrier results in serious complications of bleeding and sepsis. The subject method can be used to promote proliferation of gastrointestinal epithelium and thereby increase the tolerated doses for radiation and chemotherapy agents. Effective treatment of gastrointestinal diseases may be determined by several criteria, including an enteritis score, other tests well known in the art.

The subject method and compositions can also be used to treat wounds resulting from dermatological diseases, such as lesions resulting from autoimmune disorders such as psoriasis. Atopic dermatitis refers to skin trauma resulting from allergies associated with an immune response caused by allergens such as pollens, foods, dander, insect venoms and plant toxins.

With age, the epidermis thins and the skin appendages atrophy. Hair becomes sparse and sebaceous secretions decrease, with consequent susceptibility to dryness, chapping, and fissuring. The dermis diminishes with loss of elastic and collagen fibers. Moreover, keratinocyte proliferation (which is indicative of skin thickness and skin proliferative capacity) decreases with age. An increase in keratinocyte proliferation is believed to counteract skin aging, i.e., wrinkles,

thickness, elasticity and repair. According to the present invention, a proliferative form of a *hedgehog* or ptc therapeutic can be used either therapeutically or cosmetically to counteract, at least for a time, the effects of aging on skin.

5 The subject method can also be used in treatment of a wound to eye tissue. Generally, damage to corneal tissue, whether by disease, surgery or injury, may affect epithelial and/or endothelial cells, depending on the nature of the wound. Corneal epithelial cells are the non-keratinized epithelial cells lining the external surface of the cornea and provide a protective barrier against the external environment. Corneal wound healing has been of concern to both clinicians and researchers. Opthomologists are frequently confronted with corneal dystrophies and problematic injuries that result in persistent and recurrent epithelial erosion, often leading to permanent endothelial loss. The use of proliferative forms of the subject *hedgehog* and/or other ptc therapeutics can be used in these instances to promote epithelialization of the affected corneal tissue.

15 To further illustrate, specific disorders typically associated with epithelial cell damage in the eye, and for which the subject method can provide beneficial treatment, include persistent corneal epithelial defects, recurrent erosions, neurotrophic corneal ulcers, keratoconjunctivitis sicca, microbial corneal ulcers, viral cornea ulcers, and the like. Surgical procedures typically causing injury to the epithelial cell layers include laser procedures performed on the ocular surface, any refractive surgical procedures such as radial keratotomy and astigmatic keratotomy, 20 conjunctival flaps, conjunctival transplants, epikeratoplasty, and corneal scraping. Moreover, superficial wounds such as scrapes, surface erosion, inflammation, etc. can cause lose of epithelial cells. According to the present invention, the corneal epithelium is contacted with an amount of a ptc or *hedgehog* therapeutic effective to cause proliferation of the corneal epithelial cells to appropriately heal the wound.

25 In other embodiments, antiproliferative preparations of *hedgehog* or ptc therapeutics can be used to inhibit lens epithelial cell proliferation to prevent post-operative complications of extracapsular cataract extraction. Cataract is an intractable eye disease and various studies on a treatment of cataract have been made. But at present, the treatment of cataract is attained by surgical operations. Cataract surgery has been applied for a long time and various operative methods have been examined. Extracapsular lens extraction has become the method of choice for removing cataracts. The major medical advantages of this technique over intracapsular extraction are lower incidence of aphakic cystoid macular edema and retinal detachment. Extracapsular extraction is also required for implantation of posterior chamber type intraocular lenses which are now considered to be the lenses of choice in most cases.

However, a disadvantage of extracapsular cataract extraction is the high incidence of posterior lens capsule opacification, often called after-cataract, which can occur in up to 50% of cases within three years after surgery. After-cataract is caused by proliferation of equatorial and anterior capsule lens epithelial cells which remain after extracapsular lens extraction. These cells proliferate to cause Sommerling rings, and along with fibroblasts which also deposit and occur on the posterior capsule, cause opacification of the posterior capsule, which interferes with vision. Prevention of after-cataract would be preferable to treatment. To inhibit secondary cataract formation, the subject method provides a means for inhibiting proliferation of the remaining lens epithelial cells. For example, such cells can be induced to remain quiescent by instilling a solution containing an antiproliferative hedgehog or ptc therapeutic preparation into the anterior chamber of the eye after lens removal. Furthermore, the solution can be osmotically balanced to provide minimal effective dosage when instilled into the anterior chamber of the eye, thereby inhibiting subcapsular epithelial growth with some specificity.

The subject method can also be used in the treatment of corneopathies marked by corneal epithelial cell proliferation, as for example in ocular epithelial disorders such as epithelial downgrowth or squamous cell carcinomas of the ocular surface.

The maintenance of tissues and organs *ex vivo* is also highly desirable. Tissue replacement therapy is well established in the treatment of human disease. For example, more than 40,000 corneal transplants were performed in the United States in 1996. Human epidermal cells can be grown *in vitro* and used to populate burn sites and chronic skin ulcers and other dermal wounds. The subject method can be used to accelerate the growth of epithelial tissue *in vitro*, as well as to accelerate the grafting of the cultured epithelial tissue to an animal host.

The present method can be used for improving the "take rate" of a skin graft. Grafts of epidermal tissue can, if the take rate of the graft is too long, blister and shear, decreasing the likelihood that the autograft will "take", i.e. adhere to the wound and form a basement membrane with the underlying granulation tissue. Take rates can be increased by the subject method by inducing proliferation of the keratinocytes. The method of increasing take rates comprises contacting the skin autograft with an effective wound healing amount of a hedgehog or ptc therapeutic compositions described in the method of promoting wound healing and in the method of promoting the growth and proliferation of keratinocytes, as described above.

Skin equivalents have many uses not only as a replacement for human or animal skin for skin grafting, but also as test skin for determining the effects of pharmaceutical substances and cosmetics on skin. A major difficulty in pharmacological, chemical and cosmetic testing is the difficulties in determining the efficacy and safety of the products on skin. One advantage of the

skin equivalents of the invention is their use as an indicator of the effects produced by such substances through in vitro testing on test skin.

Thus, in one embodiment of the subject method can be used as part of a protocol for skin grafting of, e.g., denuded areas, granulating wounds and burns. The use of proliferative hedgehog and/or ptc therapeutics can enhance such grafting techniques as split thickness autografts and epidermal autografts (cultured autogenic keratinocytes) and epidermal allografts (cultured allogenic keratinocytes). In the instance of the allograft, the use of the subject method to enhance the formation of skin equivalents in culture helps to provide/maintain a ready supply of such grafts (e.g., in tissue banks) so that the patients might be covered in a single procedure with a material which allows permanent healing to occur.

In this regard, the present invention also concerns composite living skin equivalents comprising an epidermal layer of cultured keratinocyte cells which have been expanded by treatment with a hedgehog or other ptc therapeutic. The subject method can be used as part of a process for the preparation of composite living skin equivalents. In an illustrative embodiment, such a method comprises obtaining a skin sample, treating the skin sample enzymically to separate the epidermis from the dermis, treating the epidermis enzymically to release the keratinocyte cells, culturing, in the presence of a hedgehog or ptc therapeutic, the epidermal keratinocytes until confluence, in parallel, or separately, treating the dermis enzymatically to release the fibroblast cells, culturing the fibroblast cells until sub-confluence, inoculating a porous, cross-linked collagen sponge membrane with the cultured fibroblast cells, incubating the inoculated collagen sponge on its surface to allow the growth of the fibroblast cells throughout the collagen sponge, and then inoculating it with cultured keratinocyte cells, and further incubating the composite skin equivalent complex in the presence of a hedgehog or ptc therapeutic to promote the growth of the cells.

In other embodiments, skin sheets containing both epithelial and mesenchymal layers can be isolated in culture and expanded with culture media supplemented with a proliferative form of a hedgehog or ptc therapeutic.

Any skin sample amenable to cell culture techniques can be used in accordance with the present invention. The skin samples may be autogenic or allogenic.

In another aspect of the invention, the subject method can be used in conjunction with various periodontal procedures in which control of epithelial cell proliferation in and around periodontal tissue is desired.

In one embodiment, proliferative forms of the hedgehog and ptc therapeutics can be used to enhance reepithelialization around natural and prosthetic teeth, e.g., to promote formation of gum tissue.

In another embodiment, antiproliferative ptc therapeutics can find application in the treatment of periodontal disease. It is estimated that in the United States alone, there are in excess of 125 million adults with periodontal disease in varying forms. Periodontal disease starts as inflammatory lesions because of specific bacteria localizing in the area where the gingiva attaches to the tooth. Usually first to occur is a vascular change in the underlying connective tissue. Inflammation in the connective tissue stimulates the following changes in the epithelial lining of the sulcus and in the epithelial attachment: increased mitotic activity in the basal epithelial layer; increased producing of keratin with desquamation; cellular desquamation adjacent to the tooth surface tends to deepen the pocket; epithelial cells of the basal layer at the bottom of the sulcus and in the area of attachment proliferate into the connective tissue and break up of the gingival fibers begins to occur, wherein dissolution of the connective tissue results in the formation of an open lesion. The application of hedgehog preparations to the periodontium can be used to inhibit proliferation of epithelial tissue and thus prevent further periodontoclastic development.

In yet another aspect, the subject method can be used to help control guided tissue regeneration, such as when used in conjunction with bioresorbable materials. For example, incorporation of periodontal implants, such as prosthetic teeth, can be facilitated by the instant method. Reattachment of a tooth involves both formation of connective tissue fibers and re-epithelization of the tooth pocket. The subject method treatment can be used to accelerate tissue reattachment by controlling the mitotic function of basal epithelial cells in early stages of wound healing.

Yet another aspect of the present invention relates to the use of hedgehog therapeutic preparations to control hair growth. Hair is basically composed of keratin, a tough and insoluble protein; its chief strength lies in its disulphide bond of cystine. Each individual hair comprises a cylindrical shaft and a root, and is contained in a follicle, a flask-like depression in the skin. The bottom of the follicle contains a finger-like projection termed the papilla, which consists of connective tissue from which hair grows, and through which blood vessels supply the cells with nourishment. The shaft is the part that extends outwards from the skin surface, whilst the root has been described as the buried part of the hair. The base of the root expands into the hair bulb, which rests upon the papilla. Cells from which the hair is produced grow in the bulb of the follicle; they are extruded in the form of fibers as the cells proliferate in the follicle. Hair "growth" refers to the formation and elongation of the hair fiber by the dividing cells.

As is well known in the art, the common hair cycle is divided into three stages: anagen, catagen and telogen. During the active phase (anagen), the epidermal stem cells of the dermal papilla divide rapidly. Daughter cells move upward and differentiate to form the concentric layers of the hair itself. The transitional stage, catagen, is marked by the cessation of mitosis of the stem cells in the follicle. The resting stage is known as telogen, where the hair is retained within the scalp for several weeks before an emerging new hair developing below it dislodges the telogen-phase shaft from its follicle. From this model it has become clear that the larger the pool of dividing stem cells that differentiate into hair cells, the more hair growth occurs. Accordingly, methods for increasing or reducing hair growth can be carried out by potentiating or inhibiting, respectively, the proliferation of these stem cells.

In one embodiment, the subject method provides a means for altering the dynamics of the hair growth cycle to induce proliferation of hair follicle cells, particularly stem cells of the hair follicle. The subject compositions and method can be used to increase hair follicle size and the rate of hair growth in warm-blooded animals, such as humans, e.g., by promoting proliferation of hair follicle stem cells. In one embodiment, the method comprises administering to the skin in the area in which hair growth is desired an amount of hedgehog or ptc therapeutic sufficient to increase hair follicle size and/or the rate of hair growth in the animal. Typically, the composition will be administered topically as a cream, and will be applied on a daily basis until hair growth is observed and for a time thereafter sufficient to maintain the desired amount of hair growth. This method can have applications in the promotion of new hair growth or stimulation of the rate of hair growth, e.g., following chemotherapeutic treatment or for treating various forms of alopecia, e.g., male pattern baldness. For instance, one of several biochemical cellular and molecular disturbances that occur during the anagen phase or catagen phase of subjects with androgenic alopecia can be corrected or improved by treatment using the subject method, e.g., in the functioning or formation of the stem cells, their migration process or during the mitosis phase of keratin production within the follicular papilla and matrix.

In other embodiments, certain of the hedgehog and ptc therapeutics (e.g., antiproliferative forms) can be employed as a way of reducing the growth of human hair as opposed to its conventional removal by cutting, shaving, or depilation. For instance, the present method can be used in the treatment of trichosis characterized by abnormally rapid or dense growth of hair, e.g. hypertrichosis. In an exemplary embodiment, hedgehog antagonists can be used to manage hirsutism, a disorder marked by abnormal hairiness. The subject method can also provide a process for extending the duration of depilation.

Moreover, because a hedgehog antagonist (or ptc agonist) will often be cytostatic to epithelial cells, rather than cytotoxic, such agents can be used to protect hair follicle cells from

cytotoxic agents which require progression into S-phase of the cell-cycle for efficacy, e.g. radiation-induced death. Treatment by the subject method can provide protection by causing the hair follicle cells to become quiescent, e.g., by inhibiting the cells from entering S phase, and thereby preventing the follicle cells from undergoing mitotic catastrophe or programmed cell death. For instance, hedgehog antagonists can be used for patients undergoing chemo- or radiation-therapies which ordinarily result in hair loss. By inhibiting cell-cycle progression during such therapies, the subject treatment can protect hair follicle cells from death which might otherwise result from activation of cell death programs. After the therapy has concluded, the hedgehog or ptc treatment can also be removed with concomitant relief of the inhibition of follicle cell proliferation.

The subject method can also be used in the treatment of folliculitis, such as folliculitis decalvans, folliculitis ulerythematososa reticulata or keloid folliculitis. For example, a cosmetic preparation of an hedgehog therapeutic can be applied topically in the treatment of pseudofolliculitis, a chronic disorder occurring most often in the submandibular region of the neck and associated with shaving, the characteristic lesions of which are erythematous papules and pustules containing buried hairs.

In another aspect of the invention, antiproliferative forms of the subject *hedgehog* and *ptc* therapeutics can be used to induce differentiation of epithelially-derived tissue. Such forms of these molecules can provide a basis for differentiation therapy for the treatment of hyperplastic and/or neoplastic conditions involving epithelial tissue. For example, such preparations can be used for the treatment of cutaneous diseases in which there is abnormal proliferation or growth of cells of the skin.

For instance, the pharmaceutical preparations of the invention are intended for the treatment of hyperplastic epidermal conditions, such as keratosis, as well as for the treatment of neoplastic epidermal conditions such as those characterized by a high proliferation rate for various skin cancers, as for example basal cell carcinoma or squamous cell carcinoma. The subject method can also be used in the treatment of autoimmune diseases affecting the skin, in particular, of dermatological diseases involving morbid proliferation and/or keratinization of the epidermis, as for example, caused by psoriasis or atopic dermatosis.

Many common diseases of the skin, such as psoriasis, squamous cell carcinoma, keratoacanthoma and actinic keratosis are characterized by localized abnormal proliferation and growth. For example, in psoriasis, which is characterized by scaly, red, elevated plaques on the skin, the keratinocytes are known to proliferate much more rapidly than normal and to differentiate less completely.

10

15

20

35



erythema, dry, moist, or greasy scaling, and yellow crusted patches on various areas, especially the scalp, with exfoliation of an excessive amount of dry scales stasis dermatitis, an often chronic, usually eczematous dermatitis. Actinic dermatitis is dermatitis that due to exposure to actinic radiation such as that from the sun, ultraviolet waves or x- or gamma-radiation.

- 5 According to the present invention, the subject hedgehog or ptc therapeutic preparations can be used in the treatment and/or prevention of certain symptoms of dermatitis caused by unwanted proliferation of epithelial cells. Such therapies for these various forms of dermatitis can also include topical and systemic corticosteroids, antipuritics, and antibiotics.

- 10 Also included in ailments which may be treated by the subject method are disorders specific to non-humans, such as mange.

#### IV. Exemplary hedgehog therapeutic compounds.

- 15 The *hedgehog* therapeutic compositions of the subject method can be generated by any of a variety of techniques, including purification of naturally occurring proteins, recombinantly produced proteins and synthetic chemistry. Polypeptide forms of the hedgehog therapeutics are preferably derived from vertebrate hedgehog proteins, e.g., have sequences corresponding to naturally occurring hedgehog proteins, or fragments thereof, from vertebrate organisms. However, it will be appreciated that the hedgehog polypeptide can correspond to a hedgehog protein (or fragment thereof) which occurs in any metazoan organism.

- 20 The various naturally-occurring *hedgehog* proteins from which the subject therapeutics can be derived are characterized by a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. *et al.* (1992) *Cell* 71:33-50; Tabata, T. *et al.* (1992) *Genes Dev.* 2635-2645; Chang, D.E. *et al.* (1994) *Development* 120:3339-3353), *hedgehog* precursor proteins naturally  
25 undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) *Science* 266:1528-1537; Porter *et al.* (1995) *Nature* 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) *supra*; Tabata *et al.* (1992) *supra*; Chang *et al.* (1994) *supra*; Lee *et al.* (1994) *supra*; Bumcrot, D.A., *et al.* (1995) *Mol. Cell. Biol.* 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) *Curr. Biol.* 5:944-955; Lai, C.J. *et al.* (1995) *Development* 121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra*; Mart, E. *et al.* (1995) *Development* 121:2537-2547; Roelink, H. *et al.* (1995) *Cell* 81:445-455). Cell surface retention of the N-

terminal peptide is dependent on autocleavage, as a truncated form of *hedgehog* encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) *Cell* 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the *hedgehog* precursor protein proceeds through an internal thioester intermediate which subsequently is cleaved in a nucleophilic substitution. It is suggested that the nucleophile is a small lipophilic molecule, more particularly cholesterol, which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), tethering it to the cell surface.

The vertebrate family of *hedgehog* genes includes at least four members, e.g., paralogs of the single drosophila *hedgehog* gene (SEQ ID No. 19). Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle *hedgehog* (*Thh*), appears specific to fish. According to the appended sequence listing, (see also Table 1) a chicken *Shh* polypeptide is encoded by SEQ ID No:1; a mouse *Dhh* polypeptide is encoded by SEQ ID No:2; a mouse *Ihh* polypeptide is encoded by SEQ ID No:3; a mouse *Shh* polypeptide is encoded by SEQ ID No:4 a zebrafish *Shh* polypeptide is encoded by SEQ ID No:5; a human *Shh* polypeptide is encoded by SEQ ID No:6; a human *Ihh* polypeptide is encoded by SEQ ID No:7; a human *Dhh* polypeptide is encoded by SEQ ID No. 8; and a zebrafish *Thh* is encoded by SEQ ID No. 9.

Table 1  
Guide to *hedgehog* sequences in Sequence Listing

	Nucleotide	Amino Acid
Chicken <i>Shh</i>	SEQ ID No. 1	SEQ ID No. 10
Mouse <i>Dhh</i>	SEQ ID No. 2	SEQ ID No. 11
Mouse <i>Ihh</i>	SEQ ID No. 3	SEQ ID No. 12
Mouse <i>Shh</i>	SEQ ID No. 4	SEQ ID No. 13
Zebrafish <i>Shh</i>	SEQ ID No. 5	SEQ ID No. 14
Human <i>Shh</i>	SEQ ID No. 6	SEQ ID No. 15
Human <i>Ihh</i>	SEQ ID No. 7	SEQ ID No. 16
Human <i>Dhh</i>	SEQ ID No. 8	SEQ ID No. 17
Zebrafish <i>Thh</i>	SEQ ID No. 9	SEQ ID No. 18
Drosophila <i>HH</i>	SEQ ID No. 19	SEQ ID No. 20

In addition to the sequence variation between the various *hedgehog* homologs, the *hedgehog* proteins are apparently present naturally in a number of different forms, including a pro-form, a full-length mature form, and several processed fragments thereof. The pro-form

includes an N-terminal signal peptide for directed secretion of the extracellular domain, while the full-length mature form lacks this signal sequence.

As described above, further processing of the mature form occurs in some instances to yield biologically active fragments of the protein. For instance, *sonic hedgehog* undergoes  
5 additional proteolytic processing to yield two peptides of approximately 19 kDa and 27 kDa, the 19kDa fragment corresponding to an proteolytic N-terminal portion of the mature protein.

In addition to proteolytic fragmentation, the vertebrate *hedgehog* proteins can also be modified post-translationally, such as by glycosylation and/or addition of lipophilic moieties, such as stents, fatty acids, etc., though bacterially produced (e.g. unmodified) forms of the  
10 proteins still maintain certain of the bioactivities of the native protein. Bioactive fragments of *hedgehog* polypeptides of the present invention have been generated and are described in great detail in, e.g., PCT publications WO 95/18856 and WO 96/17924.

There are a wide range of lipophilic moieties with which *hedgehog* polypeptides can be derivatived. The term "lipophilic group", in the context of being attached to a *hedgehog*  
15 polypeptide, refers to a group having high hydrocarbon content thereby giving the group high affinity to lipid phases. A lipophilic group can be, for example, a relatively long chain alkyl or cycloalkyl (preferably n-alkyl) group having approximately 7 to 30 carbons. The alkyl group may terminate with a hydroxy or primary amine "tail". To further illustrate, lipophilic molecules include naturally-occurring and synthetic aromatic and non-aromatic moieties such as fatty acids,  
20 sterols, esters and alcohols, other lipid molecules, cage structures such as adamantane and buckminsterfullerenes, and aromatic hydrocarbons such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.

In one embodiment, the *hedgehog* polypeptide is modified with one or more sterol moieties, such as cholesterol. See, for example, PCT publication WO 96/17924. In certain  
25 embodiments, the cholesterol is preferably added to the C-terminal glycine were the *hedgehog* polypeptide corresponds to the naturally-occurring N-terminal proteolytic fragment.

In another embodiment, the *hedgehog* polypeptide can be modified with a fatty acid moiety, such as a myrostoyl, palmitoyl, stearoyl, or arachidoyl moiety. See, e.g., Pepinsky et al. (1998) J Biol. Chem 273: 14037.

30 In addition to those effects seen by cholesterol-addition to the C-terminus or fatty acid addition to the N-terminus of extracellular fragments of the protein, at least certain of the biological activities of the *hedgehog* gene products are unexpectedly potentiated by derivatization of the protein with lipophilic moieties at other sites on the protein and/or by moieties other than cholesterol or fatty acids. Certain aspects of the invention are directed to the

use of preparations of hedgehog polypeptides which are modified at sites other than N-terminal or C-terminal residues of the natural processed form of the protein, and/or which are modified at such terminal residues with lipophilic moieties other than a sterol at the C-terminus or fatty acid at the N-terminus.

5            Particularly useful as lipophilic molecules are alicyclic hydrocarbons, saturated and unsaturated fatty acids and other lipid and phospholipid moieties, waxes, cholesterol, isoprenoids, terpenes and polyalicyclic hydrocarbons including adamantane and buckminsterfullerenes, vitamins, polyethylene glycol or oligoethylene glycol, (C1-C18)-alkyl phosphate diesters, -O-CH<sub>2</sub>-CH(OH)-O-(C12-C18)-alkyl, and in particular conjugates with  
10 pyrene derivatives. The lipophilic moiety can be a lipophilic dye suitable for use in the invention include, but are not limited to, diphenylhexatriene, Nile Red, N-phenyl-1-naphthylamine, Prodan, Laurodan, Pyrene, Perylene, rhodamine, rhodamine B, tetramethylrhodamine, Texas Red, sulforhodamine, 1,1'-didodecyl-3,3',3'tetramethylindocarbocyanine perchlorate, octadecyl rhodamine B and the BODIPY dyes available from Molecular Probes Inc.

15            Other exemplary lipophilic moieties include aliphatic carbonyl radical groups include 1- or 2-adamantylacetyl, 3-methyladamant-1-ylacetyl, 3-methyl-3-bromo-1-adamantylacetyl, 1-decalinacetyl, camphoracetyl, camphaneacetyl, noradamantylacetyl, norbornaneacetyl, bicyclo[2.2.2.]-oct-5-eneacetyl, 1-methoxybicyclo[2.2.2.]-oct-5-ene-2-carbonyl, cis-5-norbornene-endo-2,3-dicarbonyl, 5-norbornen-2-ylacetyl, (1R)-( - )-myrtenaneacetyl, 2-norbornaneacetyl, anti-3-oxo-tricyclo[2.2.1.0<2,6>]-heptane-7-carbonyl, decanoyl, dodecanoyl, dodecenoyl, tetradecadienoyl, decynoyl or dodecynoyl.  
20

The hedgehog polypeptide can be linked to the hydrophobic moiety in a number of ways including by chemical coupling means, or by genetic engineering.

25            There are a large number of chemical cross-linking agents that are known to those skilled in the art. For the present invention, the preferred cross-linking agents are heterobifunctional cross-linkers, which can be used to link the hedgehog polypeptide and hydrophobic moiety in a stepwise manner. Heterobifunctional cross-linkers provide the ability to design more specific coupling methods for conjugating to proteins, thereby reducing the occurrences of unwanted side reactions such as homo-protein polymers. A wide variety of heterobifunctional cross-linkers are  
30 known in the art. These include: succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-succinimidylloxycarbonyl- $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)-toluene (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate  
35

(LC-SPDP). Those cross-linking agents having N-hydroxysuccinimide moieties can be obtained as the N-hydroxysulfosuccinimide analogs, which generally have greater water solubility. In addition, those cross-linking agents having disulfide bridges within the linking chain can be synthesized instead as the alkyl derivatives so as to reduce the amount of linker cleavage *in vivo*.

5 In addition to the heterobifunctional cross-linkers, there exists a number of other cross-linking agents including homobifunctional and photoreactive cross-linkers. Disuccinimidyl suberate (DSS), bismaleimido-hexane (BMH) and dimethylpimelimidate-2 HCl (DMP) are examples of useful homobifunctional cross-linking agents, and bis-[ $\beta$ -(4-azidosalicylamido)ethyl]disulfide (BASED) and N-succinimidyl-6(4'-azido-2'-nitrophenyl-10 amino)hexanoate (SANPAH) are examples of useful photoreactive cross-linkers for use in this invention. For a recent review of protein coupling techniques, see Means et al. (1990) *Bioconjugate Chemistry* 1:2-12, incorporated by reference herein.

15 One particularly useful class of heterobifunctional cross-linkers, included above, contain the primary amine reactive group, N-hydroxysuccinimide (NHS), or its water soluble analog N-hydroxysulfosuccinimide (sulfo-NHS). Primary amines (lysine epsilon groups) at alkaline pH's are unprotonated and react by nucleophilic attack on NHS or sulfo-NHS esters. This reaction results in the formation of an amide bond, and release of NHS or sulfo-NHS as a by-product.

20 Another reactive group useful as part of a heterobifunctional cross-linker is a thiol reactive group. Common thiol reactive groups include maleimides, halogens, and pyridyl disulfides. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.5-7.5) conditions. Halogens (iodoacetyl functions) react with -SH groups at physiological pH's. Both of these reactive groups result in the formation of stable thioether bonds.

25 The third component of the heterobifunctional cross-linker is the spacer arm or bridge. The bridge is the structure that connects the two reactive ends. The most apparent attribute of the bridge is its effect on steric hindrance. In some instances, a longer bridge can more easily span the distance necessary to link two complex biomolecules. For instance, SMPB has a span of 14.5 angstroms.

30 Preparing protein-protein conjugates using heterobifunctional reagents is a two-step process involving the amine reaction and the sulfhydryl reaction. For the first step, the amine reaction, the protein chosen should contain a primary amine. This can be lysine epsilon amines or a primary alpha amine found at the N-terminus of most proteins. The protein should not contain free sulfhydryl groups. In cases where both proteins to be conjugated contain free sulfhydryl groups, one protein can be modified so that all sulfhydryls are blocked using for

instance, N-ethylmaleimide (see Partis et al. (1983) J. Pro. Chem. 2:263, incorporated by reference herein). Ellman's Reagent can be used to calculate the quantity of sulfhydryls in a particular protein (see for example Ellman et al. (1958) Arch. Biochem. Biophys. 74:443 and Riddles et al. (1979) Anal. Biochem. 94:75, incorporated by reference herein).

5       The reaction buffer should be free of extraneous amines and sulfhydryls. The pH of the reaction buffer should be 7.0-7.5. This pH range prevents maleimide groups from reacting with amines, preserving the maleimide group for the second reaction with sulfhydryls.

10       The NHS-ester containing cross-linkers have limited water solubility. They should be dissolved in a minimal amount of organic solvent (DMF or DMSO) before introducing the cross-linker into the reaction mixture. The cross-linker/solvent forms an emulsion which will allow the reaction to occur.

15       The sulfo-NHS ester analogs are more water soluble, and can be added directly to the reaction buffer. Buffers of high ionic strength should be avoided, as they have a tendency to "salt out" the sulfo-NHS esters. To avoid loss of reactivity due to hydrolysis, the cross-linker is added to the reaction mixture immediately after dissolving the protein solution.

20       The reactions can be more efficient in concentrated protein solutions. The more alkaline the pH of the reaction mixture, the faster the rate of reaction. The rate of hydrolysis of the NHS and sulfo-NHS esters will also increase with increasing pH. Higher temperatures will increase the reaction rates for both hydrolysis and acylation.

25       Once the reaction is completed, the first protein is now activated, with a sulfhydryl reactive moiety. The activated protein may be isolated from the reaction mixture by simple gel filtration or dialysis. To carry out the second step of the cross-linking, the sulfhydryl reaction, the lipophilic group chosen for reaction with maleimides, activated halogens, or pyridyl disulfides must contain a free sulfhydryl. Alternatively, a primary amine may be modified with to add a sulfhydryl

30       In all cases, the buffer should be degassed to prevent oxidation of sulfhydryl groups. EDTA may be added to chelate any oxidizing metals that may be present in the buffer. Buffers should be free of any sulfhydryl containing compounds.

30       Maleimides react specifically with -SH groups at slightly acidic to neutral pH ranges (6.5-7.5). A neutral pH is sufficient for reactions involving halogens and pyridyl disulfides. Under these conditions, maleimides generally react with -SH groups within a matter of minutes. Longer reaction times are required for halogens and pyridyl disulfides.

The first sulfhydryl reactive-protein prepared in the amine reaction step is mixed with the sulfhydryl-containing lipophilic group under the appropriate buffer conditions. The conjugates can be isolated from the reaction mixture by methods such as gel filtration or by dialysis.

Exemplary activated lipophilic moieties for conjugation include: N-(1-pyrene)maleimide; 5 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide; fluorescein-5-maleimide; N-(4-(6-dimethylamino- 2-benzofuranyl)phenyl)maleimide; benzophenone-4-maleimide; 4-dimethylaminophenylazophenyl- 4'-maleimide (DABMI), tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine Red™ C2 maleimide, N-(5-aminopentyl)maleimide, trifluoroacetic acid salt, N-(2-aminoethyl)maleimide, trifluoroacetic acid salt, Oregon Green™ 488 maleimide, N-(2-(((4-azido- 2,3,5,6-tetrafluoro)benzoyl) amino)ethyl)dithio)ethyl)maleimide (TFPAM-SS1), 2-(1-(3-dimethylaminopropyl) -indol-3-yl)- 3-(indol-3-yl) maleimide (bisindolylmaleimide; GF 109203X), BODIPY® FL N-(2-aminoethyl)maleimide, N-(7-dimethylamino- 4-methylcoumarin-3-yl)maleimide (DACM), Alexa™ 488 C5 maleimide, Alexa™ 594 C5 maleimide, sodium salt N-(1-pyrene)maleimide, 15 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide, fluorescein-5-maleimide, N-(4-(6-dimethylamino- 2-benzofuranyl)phenyl)maleimide, benzophenone-4-maleimide, 4-dimethylaminophenylazophenyl- 4'-maleimide, 1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2- yl)pyridinium methanesulfonate, tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine Red™ C2 maleimide, N-(5-aminopentyl)maleimide, N-(2-aminoethyl)maleimide, N-(2-(((4-azido- 2,3,5,6-tetrafluoro)benzoyl) amino)ethyl)dithio)ethyl)maleimide, 2-(1-(3-dimethylaminopropyl) -indol-3-yl)-3-(indol-3-yl) maleimide, N-(7-dimethylamino- 4-methylcoumarin-3-yl)maleimide (DACM), 11H-Benzo[a]fluorene, Benzo[a]pyrene. 20

In one embodiment, the hedgehog polypeptide can be derivatized using pyrene maleimide, which can be purchased from Molecular Probes (Eugene, Oreg.), e.g., N-(1-pyrene)maleimide or 1-pyrenemethyl iodoacetate (PMIA ester). 25

For those embodiments wherein the hydrophobic moiety is a polypeptide, the modified hedgehog polypeptide of this invention can be constructed as a fusion protein, containing the hedgehog polypeptide and the hydrophobic moiety as one contiguous polypeptide chain.

30 In certain embodiments, the lipophilic moiety is an amphipathic polypeptide, such as magainin, cecropin, attacin, melittin, gramicidin S, alpha-toxin of Staph. aureus, alamethicin or a synthetic amphipathic polypeptide. Fusogenic coat proteins from viral particles can also be a convenient source of amphipathic sequences for the subject hedgehog proteins

Moreover, mutagenesis can be used to create modified *hh* polypeptides, e.g., for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. Modified *hedgehog* polypeptides can also include those with altered post-translational processing relative to a naturally occurring *hedgehog* protein, e.g., altered glycosylation, cholesterolization, prenylation and the like.

In one embodiment, the *hedgehog* therapeutic is a polypeptide encodable by a nucleotide sequence that hybridizes under stringent conditions to a *hedgehog* coding sequence represented in one or more of SEQ ID Nos:1-7. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

As described in the literature, genes for other *hedgehog* proteins, e.g., from other animals, can be obtained from mRNA or genomic DNA samples using techniques well known in the art. For example, a cDNA encoding a *hedgehog* protein can be obtained by isolating total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding a *hedgehog* protein can also be cloned using established polymerase chain reaction techniques.

Preferred nucleic acids encode a *hedgehog* polypeptide comprising an amino acid sequence at least 60% homologous or identical, more preferably 70% homologous or identical, and most preferably 80% homologous or identical with an amino acid sequence selected from the group consisting of SEQ ID Nos:8-14. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology or identity with an amino acid sequence represented in one of SEQ ID Nos:8-14 are also within the scope of the invention.

In addition to native *hedgehog* proteins, *hedgehog* polypeptides preferred by the present invention are at least 60% homologous or identical, more preferably 70% homologous or identical and most preferably 80% homologous or identical with an amino acid sequence represented by any of SEQ ID Nos:8-14. Polypeptides which are at least 90%, more preferably



at least 95%, and most preferably at least about 98-99% homologous or identical with a sequence selected from the group consisting of SEQ ID Nos:8-14 are also within the scope of the invention. The only prerequisite is that the *hedgehog* polypeptide is capable of modulating the growth of epithelial cells.

5       The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a *hedgehog* polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to  
10 recombinant *hedgehog* gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native *hedgehog* protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

The method of the present invention can also be carried out using variant forms of the naturally occurring *hedgehog* polypeptides, e.g., mutational variants.

15       As is known in the art, *hedgehog* polypeptides can be produced by standard biological techniques or by chemical synthesis. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The polypeptide *hedgehog* may be secreted and isolated from a mixture of cells and medium  
20 containing the recombinant *hedgehog* polypeptide. Alternatively, the peptide may be retained cytoplasmically by removing the signal peptide sequence from the recombinant *hedgehog* gene and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant *hedgehog* polypeptide can be isolated from cell culture medium, host cells, or both using  
25 techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant *hedgehog* polypeptide is a fusion protein containing a domain which facilitates its purification, such as an *hedgehog*/GST fusion protein. The host cell may be any prokaryotic or eukaryotic cell.

30       Recombinant *hedgehog* genes can be produced by ligating nucleic acid encoding an *hedgehog* protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject *hedgehog* polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a *hedgehog* polypeptide include plasmids of the types: pBR322-derived

plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

5 A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles  
10 useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due to the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative  
15 embodiment, an *hedgehog* polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of one of the *hedgehog* genes represented in SEQ ID Nos:1-7.

20 The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNA1/amp, pcDNA1/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively,  
25 derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

30 In some instances, it may be desirable to express the recombinant *hedgehog* polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the  $\beta$ -gal containing pBlueBac III).

35 When it is desirable to express only a portion of an *hedgehog* protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal

position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) *PNAS* 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing *hedgehog*-derived polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., *supra*).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. It is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the *hedgehog* polypeptides of the present invention. For example, *hedgehog* polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the *hedgehog* polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence, can be used to replace the signal sequence which naturally occurs at the N-terminus of the *hedgehog* protein (e.g. of the pro-form, in order to permit purification of the poly(His)-*hedgehog* protein by affinity chromatography using a Ni<sup>2+</sup> metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

*Hedgehog* polypeptides may also be chemically modified to create *hedgehog* derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl

groups, cholesterol, isoprenoids, lipids, phosphate, acetyl groups and the like. Covalent derivatives of *hedgehog* proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

5 For instance, *hedgehog* proteins can be generated to include a moiety, other than sequence naturally associated with the protein, that binds a component of the extracellular matrix and enhances localization of the analog to cell surfaces. For example, sequences derived from the fibronectin "type-III repeat", such as a tetrapeptide sequence R-G-D-S (Pierschbacher et al. (1984) *Nature* 309:30-3; and Kornblihtt et al. (1985) *EMBO* 4:1755-9) can be added to the  
10 *hedgehog* polypeptide to support attachment of the chimeric molecule to a cell through binding ECM components (Ruoslahti et al. (1987) *Science* 238:491-497; Pierschbacher et al. (1987) *J. Biol. Chem.* 262:17294-8.; Hynes (1987) *Cell* 48:549-54; and Hynes (1992) *Cell* 69:11-25).

In a preferred embodiment, the *hedgehog* polypeptide is isolated from, or is otherwise substantially free of, other cellular proteins, especially other extracellular or cell surface  
15 associated proteins which may normally be associated with the *hedgehog* polypeptide, unless provided in the form of fusion protein with the *hedgehog* polypeptide. The term "substantially free of other cellular or extracellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure preparations" or "purified preparations" are defined as encompassing preparations of *hedgehog* polypeptides having less than 20% (by dry weight)  
20 contaminating protein, and preferably having less than 5% contaminating protein. By "purified", it is meant that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water,  
25 buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in any of SEQ ID Nos:10-18 or  
30 20, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of *hedgehog* polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

With respect to bioactive fragments of *hedgehog* polypeptide, preferred *hedgehog*  
35 therapeutics include at least 50 (contiguous) amino acid residues of a *hedgehog* polypeptide,

more preferably at least 100 (contiguous), and even more preferably at least 150 (contiguous) residues.

Another preferred *hedgehog* polypeptide which can be included in the *hedgehog* therapeutic is an N-terminal fragment of the mature protein having a molecular weight of approximately 19 kDa.

Preferred human *hedgehog* proteins include N-terminal fragments corresponding approximately to residues 24-197 of SEQ ID No. 15, 28-202 of SEQ ID No. 16, and 23-198 of SEQ ID No. 17. By "corresponding approximately" it is meant that the sequence of interest is at most 20 amino acid residues different in length to the reference sequence, though more preferably at most 5, 10 or 15 amino acid different in length.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13 or SEQ ID No:14, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of hedgehog polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

Still other preferred *hedgehog* polypeptides includes an amino acid sequence represented by the formula A-B wherein: (i) A represents all or the portion of the amino acid sequence designated by residues 1-168 of SEQ ID No:21; and B represents at least one amino acid residue of the amino acid sequence designated by residues 169-221 of SEQ ID No:21; (ii) A represents all or the portion of the amino acid sequence designated by residues 24-193 of SEQ ID No:15; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:15; (iii) A represents all or the portion of the amino acid sequence designated by residues 25-193 of SEQ ID No:13; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:13; (iv) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No:11; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:11; (v) A represents all or the portion of the amino acid sequence designated by residues 28-197 of SEQ ID No:12; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:12; (vi) A represents all or the portion of the amino acid sequence designated by residues 29-197 of SEQ ID No:16; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:16; or (vii) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No. 17, and B

represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No. 17. In certain preferred embodiments, A and B together represent a contiguous polypeptide sequence designated sequence, A represents at least 25, 50, 75, 100, 125 or 150 (contiguous) amino acids of the designated sequence, and B represents at least 5, 10, or 20 (contiguous) amino acid residues of the amino acid sequence designated by corresponding entry in the sequence listing, and A and B together preferably represent a contiguous sequence corresponding to the sequence listing entry. Similar fragments from other *hedgehog* also contemplated, e.g., fragments which correspond to the preferred fragments from the sequence listing entries which are enumerated above. In preferred embodiments, the *hedgehog* polypeptide includes a C-terminal glycine (or other appropriate residue) which is derivatized with a cholesterol.

Isolated peptidyl portions of *hedgehog* proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a *hedgehog* polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") *hedgehog* protein. For example, Román et al. (1994) *Eur J Biochem* 222:65-73 describe the use of competitive-binding assays using short, overlapping synthetic peptides from larger proteins to identify binding domains.

The recombinant *hedgehog* polypeptides of the present invention also include homologs of the authentic *hedgehog* proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter potential cleavage sequences or which inactivate an enzymatic activity associated with the protein. *Hedgehog* homologs of the present invention also include proteins which have been post-translationally modified in a manner different than the authentic protein. Exemplary derivatives of *hedgehog* proteins include polypeptides which lack N-glycosylation sites (e.g. to produce an unglycosylated protein), which lack sites for cholesterolization, and/or which lack N-terminal and/or C-terminal sequences.

Modification of the structure of the subject *hedgehog* polypeptides can also be for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the *hedgehog* polypeptides described in more detail herein. Such

modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

It is well known in the art that one could reasonably expect that certain isolated replacements of amino acids, e.g., replacement of an amino acid residue with another related amino acid (i.e. isosteric and/or isoelectric mutations), can be carried out without major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur-containing = cysteine and methionine. (see, for example, *Biochemistry*, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional *hedgehog* homolog (e.g. functional in the sense that it acts to mimic or antagonize the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

It is specifically contemplated that the methods of the present invention can be carried using homologs of naturally occurring hedgehog proteins. In one embodiment, the invention contemplates using hedgehog polypeptides generated by combinatorial mutagenesis. Such methods, as are known in the art, are convenient for generating both point and truncation mutants, and can be especially useful for identifying potential variant sequences (e.g. homologs) that are functional in binding to a receptor for *hedgehog* proteins. The purpose of screening such combinatorial libraries is to generate, for example, novel *hedgehog* homologs which can act as either agonists or antagonist. To illustrate, *hedgehog* homologs can be engineered by the present method to provide more efficient binding to a cognate receptor, such as *patched*, yet still retain at least a portion of an activity associated with *hedgehog*. Thus, combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein. Likewise, *hedgehog* homologs can be generated by the present combinatorial approach

to act as antagonists, in that they are able to mimic, for example, binding to other extracellular matrix components (such as receptors), yet not induce any biological response, thereby inhibiting the action of authentic *hedgehog* or *hedgehog* agonists. Moreover, manipulation of certain domains of *hedgehog* by the present method can provide domains more suitable for use in fusion proteins, such as one that incorporates portions of other proteins which are derived from the extracellular matrix and/or which bind extracellular matrix components.

To further illustrate the state of the art of combinatorial mutagenesis, it is noted that the review article of Gallop et al. (1994) *J Med Chem* 37:1233 describes the general state of the art of combinatorial libraries as of the earlier 1990's. In particular, Gallop et al state at page 1239 "[s]creening the analog libraries aids in determining the minimum size of the active sequence and in identifying those residues critical for binding and intolerant of substitution". In addition, the Ladner et al. PCT publication WO90/02809, the Goeddel et al. U.S. Patent 5,223,408, and the Markland et al. PCT publication WO92/15679 illustrate specific techniques which one skilled in the art could utilize to generate libraries of *hedgehog* variants which can be rapidly screened to identify variants/fragments which retained a particular activity of the *hedgehog* polypeptides. These techniques are exemplary of the art and demonstrate that large libraries of related variants/truncants can be generated and assayed to isolate particular variants without undue experimentation. Gustin et al. (1993) *Virology* 193:653, and Bass et al. (1990) *Proteins: Structure, Function and Genetics* 8:309-314 also describe other exemplary techniques from the art which can be adapted as means for generating mutagenic variants of *hedgehog* polypeptides.

Indeed, it is plain from the combinatorial mutagenesis art that large scale mutagenesis of *hedgehog* proteins, without any preconceived ideas of which residues were critical to the biological function, and generate wide arrays of variants having equivalent biological activity. Indeed, it is the ability of combinatorial techniques to screen billions of different variants by high throughput analysis that removes any requirement of *a priori* understanding or knowledge of critical residues.

To illustrate, the amino acid sequences for a population of *hedgehog* homologs or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, *hedgehog* homologs from one or more species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of *hedgehog* variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *hedgehog*



sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of *hedgehog* sequences therein.

As illustrated in PCT publication WO 95/18856, to analyze the sequences of a population of variants, the amino acid sequences of interest can be aligned relative to sequence homology.

- 5 The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial.

In an illustrative embodiment, alignment of exons 1, 2 and a portion of exon 3 encoded sequences (e.g. the N-terminal approximately 221 residues of the mature protein) of each of the *Shh* clones produces a degenerate set of *Shh* polypeptides represented by the general formula:

10 C-G-P-G-R-G-X(1)-G-X(2)-R-R-H-P-K-K-L-T-P-L-A-Y-K-Q-F-I-P-N-V-A-E-  
K-T-L-G-A-S-G-R-Y-E-G-K-I-X(3)-R-N-S-E-R-F-K-E-L-T-P-N-Y-N-P-D-I-I-F-  
K-D-E-E-N-T-G-A-D-R-L-M-T-Q-R-C-K-D-K-L-N-X(4)-L-A-I-S-V-M-N-X(5)-  
W-P-G-V-X(6)-L-R-V-T-E-G-W-D-E-D-G-H-H-X(7)-E-E-S-L-H-Y-E-G-R-A-  
15 V-D-I-T-T-S-D-R-D-X(8)-S-K-Y-G-X(9)-L-X(10)-R-L-A-V-E-A-G-F-D-W-V-  
Y-Y-E-S-K-A-H-I-H-C-S-V-K-A-E-N-S-V-A-A-K-S-G-G-C-F-P-G-S-A-X(11)-  
V-X(12)-L-X(13)-X(14)-G-G-X(15)-K-X(16)-V-K-D-L-X(17)-P-G-D-X(18)-V-  
L-A-A-D-X(19)-X(20)-G-X(21)-L-X(22)-X(23)-S-D-F-X(24)-X(25)-F-X(26)-D-  
R (SEQ ID No: 21)

- 20 wherein each of the degenerate positions "X" can be an amino acid which occurs in that position in one of the human, mouse, chicken or zebrafish *Shh* clones, or, to expand the library, each X can also be selected from amongst amino acid residue which would be conservative substitutions for the amino acids which appear naturally in each of those positions. For instance, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Phe, Tyr or Trp ; Xaa(2) represents Arg, His or Lys; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(4) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(5) represents Lys, Arg, His, Asn or Gln; Xaa(6) represents Lys, Arg or His; Xaa(7) represents Ser, Thr, Tyr, Trp or Phe; Xaa(8) represents Lys, Arg or His; Xaa(9) represents Met, Cys, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Leu, Val, Met, Thr or Ser; Xaa(12) represents His, Phe, Tyr, Ser, Thr, Met or Cys; Xaa(13) represents Gln, Asn, Glu, or Asp; Xaa(14) represents His, Phe, Tyr, Thr, Gln, Asn, Glu or Asp; Xaa(15) represents Gln, Asn, Glu, Asp, Thr, Ser, Met or Cys; Xaa(16) represents Ala, Gly, Cys, Leu, Val or Met; Xaa(17) represents Arg, Lys, Met, Ile, Asn, Asp, Glu, Gln, Ser, Thr or Cys; Xaa(18) represents Arg, Lys, Met or Ile; Xaa(19) represents Ala, Gly, Cys, Asp, Glu, Gln, Asn, Ser, Thr or Met; Xaa(20) represents Ala, Gly, Cys, Asp, Asn, Glu or Gln; Xaa(21) represents Arg, Lys, Met, Ile, Asn, Asp, Glu or Gln; Xaa(22) represent Leu, Val, Met or Ile; Xaa(23) represents Phe, Tyr, Thr, His or Trp; Xaa(24) represents Ile, Val, Leu or Met; Xaa(25) represents Met, Cys, Ile,
- 25
- 30
- 35

Leu, Val, Thr or Ser; Xaa(26) represents Leu, Val, Met, Thr or Ser. In an even more expansive library, each X can be selected from any amino acid.

In similar fashion, alignment of each of the human, mouse, chicken and zebrafish *hedgehog* clones, can provide a degenerate polypeptide sequence represented by the general formula:

5 C-G-P-G-R-G-X(1)-X(2)-X(3)-R-R-X(4)-X(5)-X(6)-P-K-X(7)-L-X(8)-P-L-X(9)-  
Y-K-Q-F-X(10)-P-X(11)-X(12)-X(13)-E-X(14)-T-L-G-A-S-G-X(15)-X(16)-E-G-  
X(17)-X(18)-X(19)-R-X(20)-S-E-R-F-X(21)-X(22)-L-T-P-N-Y-N-P-D-I-I-F-K-  
10 D-E-E-N-X(23)-G-A-D-R-L-M-T-X(24)-R-C-K-X(25)-X(26)-X(27)-N-X(28)-L-  
A-I-S-V-M-N-X(29)-W-P-G-V-X(30)-L-R-V-T-E-G-X(31)-D-E-D-G-H-H-  
X(32)-X(33)-X(34)-S-L-H-Y-E-G-R-A-X(35)-D-I-T-T-S-D-R-D-X(36)-X(37)-K-  
Y-G-X(38)-L-X(39)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-X(40)-X(41)-H-X(42)-  
H-X(43)-S-V-K-X(44)-X(45) (SEQ IDNo:22)

wherein, as above, each of the degenerate positions "X" can be an amino acid which occurs in a  
15 corresponding position in one of the wild-type clones, and may also include amino acid residue  
which would be conservative substitutions, or each X can be any amino acid residue. In an  
exemplary embodiment, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Pro, Phe or Tyr; Xaa(2)  
represents Gly, Ala, Val, Leu or Ile; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Lys, His or Arg;  
Xaa(4) represents Lys, Arg or His; Xaa(5) represents Phe, Trp, Tyr or an amino acid gap; Xaa(6)  
20 represents Gly, Ala, Val, Leu, Ile or an amino acid gap; Xaa(7) represents Asn, Gln, His, Arg or  
Lys; Xaa(8) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(9) represents Gly, Ala, Val, Leu,  
Ile, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Ser,  
Thr, Gln or Asn; Xaa(12) represents Met, Cys, Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(13)  
represents Gly, Ala, Val, Leu, Ile or Pro; Xaa(14) represents Arg, His or Lys; Xaa(15) represents  
25 Gly, Ala, Val, Leu, Ile, Pro, Arg, His or Lys; Xaa(16) represents Gly, Ala, Val, Leu, Ile, Phe or  
Tyr; Xaa(17) represents Arg, His or Lys; Xaa(18) represents Gly, Ala, Val, Leu, Ile, Ser or Thr;  
Xaa(19) represents Thr or Ser; Xaa(20) represents Gly, Ala, Val, Leu, Ile, Asn or Gln; Xaa(21)  
represents Arg, His or Lys; Xaa(22) represents Asp or Glu; Xaa(23) represents Ser or Thr;  
Xaa(24) represents Glu, Asp, Gln or Asn; Xaa(25) represents Glu or Asp; Xaa(26) represents  
30 Arg, His or Lys; Xaa(27) represents Gly, Ala, Val, Leu or Ile; Xaa(28) represents Gly, Ala, Val,  
Leu, Ile, Thr or Ser; Xaa(29) represents Met, Cys, Gln, Asn, Arg, Lys or His; Xaa(30) represents  
Arg, His or Lys; Xaa(31) represents Trp, Phe, Tyr, Arg, His or Lys; Xaa(32) represents Gly, Ala,  
Val, Leu, Ile, Ser, Thr, Tyr or Phe; Xaa(33) represents Gln, Asn, Asp or Glu; Xaa(34) represents  
Asp or Glu; Xaa(35) represents Gly, Ala, Val, Leu, or Ile; Xaa(36) represents Arg, His or Lys;  
35 Xaa(37) represents Asn, Gln, Thr or Ser; Xaa(38) represents Gly, Ala, Val, Leu, Ile, Ser, Thr,  
Met or Cys; Xaa(39) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(40) represents Arg, His

or Lys; Xaa(41) represents Asn, Gln, Gly, Ala, Val, Leu or Ile; Xaa(42) represents Gly, Ala, Val, Leu or Ile; Xaa(43) represents Gly, Ala, Val, Leu, Ile, Ser, Thr or Cys; Xaa(44) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; and Xaa(45) represents Asp or Glu.

There are many ways by which the library of potential *hedgehog* homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *hedgehog* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of *hedgehog* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate *hedgehog* sequences created by combinatorial mutagenesis techniques.

In one embodiment, the combinatorial library is designed to be secreted (e.g. the polypeptides of the library all include a signal sequence but no transmembrane or cytoplasmic domains), and is used to transfect a eukaryotic cell that can be co-cultured with epithelial stem cells. A functional *hedgehog* protein secreted by the cells expressing the combinatorial library will diffuse to neighboring epithelial cells and induce a particular biological response, such as proliferation. The pattern of detection of proliferation will resemble a gradient function, and will allow the isolation (generally after several repetitive rounds of selection) of cells producing *hedgehog* homologs active as proliferative agents with respect to epithelial cells. Likewise,

*hedgehog* antagonists can be selected in similar fashion by the ability of the cell producing a functional antagonist to protect neighboring cells (e.g., to inhibit proliferation) from the effect of wild-type *hedgehog* added to the culture media.

To illustrate, target epithelial cells are cultured in 24-well microtitre plates. Other eukaryotic cells are transfected with the combinatorial *hedgehog* gene library and cultured in cell culture inserts (e.g. Collaborative Biomedical Products, Catalog #40446) that are able to fit into the wells of the microtitre plate. The cell culture inserts are placed in the wells such that recombinant *hedgehog* homologs secreted by the cells in the insert can diffuse through the porous bottom of the insert and contact the target cells in the microtitre plate wells. After a period of time sufficient for functional forms of a *hedgehog* protein to produce a measurable response in the target cells, such as proliferation, the inserts are removed and the effect of the variant *hedgehog* proteins on the target cells determined. Cells from the inserts corresponding to wells which score positive for activity can be split and re-cultured on several inserts, the process being repeated until the active clones are identified.

In yet another screening assay, the candidate *hedgehog* gene products are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to associate with a *hedgehog*-binding moiety (such as the *patched* protein or other *hedgehog* receptor) via this gene product is detected in a "panning assay". Such panning steps can be carried out on cells cultured from embryos. For instance, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, fluorescently labeled molecules which bind *hedgehog* can be used to score for potentially functional *hedgehog* homologs. Cells can be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, separated by a fluorescence-activated cell sorter.

In an alternate embodiment, the gene library is expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E.coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO

90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) J. Biol. Chem. 267:16007-16010; Griffiths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457-4461).

In an illustrative embodiment, the recombinant phage antibody system (RPAS, 5 Pharamacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening *hedgehog* combinatorial libraries. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The *hedgehog* combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to 10 transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate *hedgehog* gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate *hedgehog*, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate *hedgehog* proteins which are capable of binding an *hedgehog* receptor are selected or 15 enriched by panning. For instance, the phage library can be applied to cells which express the *patched* protein and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for *hedgehog* homologs, which can then be screened for further 20 biological activities in order to differentiate agonists and antagonists.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of  $10^{26}$  molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays such as phage display. To overcome this problem, a new technique has been developed recently, recursive 25 ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, *PNAS USA* 30 89:7811-7815; Yourvan et al., 1992, *Parallel Problem Solving from Nature*, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, *Protein Engineering* 6(3):327-331).

The invention also provides for reduction of the *hedgehog* protein to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt binding of a *hedgehog* polypeptide 35 of the present invention with an *hedgehog* receptor. Thus, such mutagenic techniques as

described above are also useful to map the determinants of the *hedgehog* proteins which participate in protein-protein interactions involved in, for example, binding of the subject *hedgehog* polypeptide to other extracellular matrix components. To illustrate, the critical residues of a subject *hedgehog* polypeptide which are involved in molecular recognition of an *hedgehog* receptor such as *patched* can be determined and used to generate *hedgehog*-derived peptidomimetics which competitively inhibit binding of the authentic *hedgehog* protein with that moiety. By employing, for example, scanning mutagenesis to map the amino acid residues of each of the subject *hedgehog* proteins which are involved in binding other extracellular proteins, peptidomimetic compounds can be generated which mimic those residues of the *hedgehog* protein which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of a *hedgehog* protein. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985),  $\beta$ -turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and  $\beta$ -aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Recombinantly produced forms of the *hedgehog* proteins can be produced using, e.g., expression vectors containing a nucleic acid encoding a *hedgehog* polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of a *hedgehog* polypeptide. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding *hedgehog* polypeptide. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the

5 TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage  $\lambda$ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast  $\alpha$ -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

10 In addition to providing a ready source of hedgehog polypeptides for purification, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of a *hedgehog* polypeptide. Thus, another aspect of the invention features expression vectors for *in vivo* transfection of a *hedgehog* polypeptide in particular cell types so as cause ectopic expression of a *hedgehog* polypeptide in an epithelial tissue.

15 Formulations of such expression constructs may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the hedgehog coding sequence in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or  $\text{CaPO}_4$  precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of *hedgehog* expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture systems described below.

20 A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the particular form of the *hedgehog* polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded

within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

5 Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding a hedgehog polypeptide and renders the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include Crip, Cre, 2 and Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and



WO94/06920). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julan et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the *hedgehog* gene of the retroviral vector.

Another viral gene delivery system useful in the present method utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they can be used to infect a wide variety of cell types, including epithelial cells (Rosenfeld et al. (1992) cited *supra*). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted *hedgehog* gene can be under control of,

for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a *hedgehog* polypeptide in the tissue of an animal.

5 Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the *hedgehog* polypeptide gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

10 In clinical settings, the gene delivery systems for the therapeutic *hedgehog* gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences  
15 controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057). A  
20 *hedgehog* expression construct can be delivered in a gene therapy construct to dermal cells by, e.g., electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which  
25 the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In yet another embodiment, the *hedgehog* or *ptc* therapeutic can be a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional  
30 regulatory sequences of an endogenous gene. For instance, the gene activation construct can replace the endogenous promoter of a *hedgehog* gene with a heterologous promoter, e.g., one which causes constitutive expression of the *hedgehog* gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. Other genes in the *patched* signaling pathway can be similarly targeted. A variety of different formats  
35 for the gene activation constructs are available. See, for example, the Transkaryotic Therapies,

Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous *hedgehog* gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic *hedgehog* gene upon recombination of the gene activation construct. For use in generating cultures of *hedgehog* producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native *hedgehog* gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous *hedgehog* gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof. Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human  $\beta$ -actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold

Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

In an exemplary embodiment, portions of the 5' flanking region of the human Shh gene are amplified using primers which add restriction sites, to generate the following fragments

10 5' -gcgcgcttcgaaGCGAGGCAGCCAGCGAGGGAGAGAGCGAGCGGGCGAGCCGGAGC-  
GAGGAAatcgatgcgcgc (primer 1)

5' -gcgcgcagatctGGGAAAGCGCAAGAGAGAGCGCACACGCACACACCCGCCGCGCG-  
CACTCGg gatccgcgcgc (primer 2)

15 As illustrated, primer 1 includes a 5' non-coding region of the human Shh gene and is flanked by an AsuII and ClaI restriction sites. Primer 2 includes a portion of the 5' non-coding region immediately 3' to that present in primer 1. The hedgehog gene sequence is flanked by XhoII and BamHI restriction sites. The purified amplimers are cut with each of the enzymes as appropriate.

20 The vector pCDNA1.1 (Invitrogen) includes a CMV promoter. The plasmid is cut with with AsuII, which cleaves just 3' to the CMV promoter sequence. The AsuII/ClaI fragment of primer 1 is ligated to the AsuII cleavage site of the pcDNA vector. The ClaI/AsuII ligation destroys the AsuII site at the 3' end of a properly inserted primer 1.

25 The vector is then cut with BamHI, and an XhoII/BamHI fragment of primer 2 is ligated to the BamHI cleavage site. As above, the BamHI/XhoII ligation destroys the BamHI site at the 5' end of a properly inserted primer 2.

30 Individual colonies are selected, cut with AsuII and BamHI, and the size of the AsuII/BamHI fragment determined. Colonies in which both the primer 1 and primer 2 sequences are correctly inserted are further amplified, an cut with AsuII and BamHI to produce the gene activation construct

cgaagcgaggcagccagcgagggagagagcgagcgggagccgagcgaggaaATCGAAGGTTC  
GAATCCTTCCCCACCACCATCAC'TTTCAAAGTCCGAAAGAATCTGCTCCCTGCTTGTGTGTTG  
GAGGTCGCTGAGTAGTGCGCGAGTAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTG

CATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGC  
GTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCA  
TATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCC  
CCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCATTGAC  
5 GTCAATGGGTGGACTATTTACGGTAAACTGCCCACCTGGCAGTACATCAAGTGTATCATATGCCA  
AGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC  
CTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGTATGC  
GGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCTCAAGTCTCCAC  
CCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCTCAAAATGTCGTAA  
10 CAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAG  
CTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGG  
GAGACCCAAGCTTGGTACCGAGCTCGGATCgatctgggaaagcgcaagagagagcgcacacgcac  
acacccgcccgcgcgcactcgg

In this construct, the flanking primer 1 and primer 2 sequences provide the recombination region  
15 which permits the insertion of the CMV promoter in front of the coding sequence for the human  
*Shh* gene. Other heterologous promoters (or other transcriptional regulatory sequences) can be  
inserted in a genomic *hedgehog* gene by a similar method.

In still other embodiments, the replacement region merely deletes a negative  
transcriptional control element of the native gene, e.g., to activate expression, or ablates a  
20 positive control element, e.g., to inhibit expression of the targeted gene.

#### V. Exemplary *ptc* therapeutic compounds.

In another embodiment, the subject method is carried out using a *ptc* therapeutic  
25 composition. Such compositions can be generated with, for example, compounds which bind to  
patched and alter its signal transduction activity, compounds which alter the binding and/or  
enzymatic activity of a protein (e.g., intracellular) involved in patched signal pathway, and  
compounds which alter the level of expression of a hedgehog protein, a patched protein or a  
protein involved in the intracellular signal transduction pathway of patched.

30 The availability of purified and recombinant *hedgehog* polypeptides facilitates the  
generation of assay systems which can be used to screen for drugs, such as small organic  
molecules, which are either agonists or antagonists of the normal cellular function of a *hedgehog*  
and/or patched protein, particularly their role in the pathogenesis of epithelial cell proliferation

and/or differentiation. In one embodiment, the assay evaluates the ability of a compound to modulate binding between a *hedgehog* polypeptide and a *hedgehog* receptor such as *patched*. In other embodiments, the assay merely scores for the ability of a test compound to alter the signal transduction activity of the *patched* protein. In this manner, a variety of *hedgehog* and/or *ptc* therapeutics, both proliferative and anti-proliferative in activity, can be identified. A variety of assay formats will suffice and, in light of the present disclosure, will be comprehended by skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with receptor proteins.

Accordingly, in an exemplary screening assay for *ptc* therapeutics, the compound of interest is contacted with a mixture including a *hedgehog* receptor protein (e.g., a cell expressing the *patched* receptor) and a *hedgehog* protein under conditions in which it is ordinarily capable of binding the *hedgehog* protein. To the mixture is then added a composition containing a test compound. Detection and quantification of receptor/*hedgehog* complexes provides a means for determining the test compound's efficacy at inhibiting (or potentiating) complex formation between the receptor protein and the *hedgehog* polypeptide. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified *hedgehog* polypeptide is added to the receptor protein, and the formation of receptor/*hedgehog* complex is quantitated in the absence of the test compound.

In other embodiments, a *ptc* therapeutic of the present invention is one which disrupts the association of *patched* with *smoothened*.

Agonist and antagonists of epithelial cell growth can be distinguished, and the efficacy of the compound can be assessed, by subsequent testing with epithelial cells, e.g., in culture.

In an illustrative embodiment, the polypeptide utilized as a *hedgehog* receptor can be generated from the *patched* protein. Accordingly, an exemplary screening assay includes all or a

5 suitable portion of the *patched* protein which can be obtained from, for example, the human *patched* gene (GenBank U43148) or other vertebrate sources (see GenBank Accession numbers U40074 for chicken *patched* and U46155 for mouse *patched*), as well as from drosophila (GenBank Accession number M28999) or other invertebrate sources. The *patched* protein can be provided in the screening assay as a whole protein (preferably expressed on the surface of a cell), or alternatively as a fragment of the full length protein which binds to *hedgehog* polypeptides, e.g., as one or both of the substantial extracellular domains (e.g. corresponding to residues Asn120-Ser438 and/or Arg770-Trp1027 of the human *patched* protein - which are also potential antagonists of *hedgehog*-dependent signal transduction). For instance, the *patched* protein can be provided in soluble form, as for example a preparation of one of the extracellular domains, or a preparation of both of the extracellular domains which are covalently connected by an unstructured linker (see, for example, Huston et al. (1988) PNAS 85:4879; and U.S. Patent No. 5,091,513). In other embodiments, the protein can be provided as part of a liposomal preparation or expressed on the surface of a cell. The *patched* protein can derived from a recombinant gene, e.g., being ectopically expressed in a heterologous cell. For instance, the protein can be expressed on oocytes, mammalian cells (e.g., COS, CHO, 3T3 or the like), or yeast cell by standard recombinant DNA techniques. These recombinant cells can be used for receptor binding, signal transduction or gene expression assays. Marigo et al. (1996) *Development* 122:1225-1233 illustrates a binding assay of human *hedgehog* to chick *patched* protein ectopically expressed in *Xenopus laevis* oocytes. The assay system of Marigo et al. can be adapted to the present drug screening assays. As illustrated in that reference, *Shh* binds to the *patched* protein in a selective, saturable, dose-dependent manner, thus demonstrating that *patched* is a receptor for *Shh*.

25 Complex formation between the *hedgehog* polypeptide and a *hedgehog* receptor may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labelled proteins such as radiolabelled, fluorescently labelled, or enzymatically labelled *hedgehog* polypeptides, by immunoassay, or by chromatographic detection.

30 Typically, for cell-free assays, it will be desirable to immobilize either the *hedgehog* receptor or the *hedgehog* polypeptide to facilitate separation of receptor/*hedgehog* complexes from uncomplexed forms of one of the proteins, as well as to accommodate automation of the assay. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/receptor (GST/receptor) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the *hedgehog*

35

polypeptide, e.g. an  $^{35}\text{S}$ -labeled *hedgehog* polypeptide, and the test compound and incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound *hedgehog* polypeptide, and the matrix bead-bound radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the receptor/*hedgehog* complexes are dissociated. Alternatively, the complexes can be dissociated from the bead, separated by SDS-PAGE gel, and the level of *hedgehog* polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, soluble portions of the *hedgehog* receptor protein can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated receptor molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the *hedgehog* receptor but which do not interfere with *hedgehog* binding can be derivatized to the wells of the plate, and the receptor trapped in the wells by antibody conjugation. As above, preparations of a *hedgehog* polypeptide and a test compound are incubated in the receptor-presenting wells of the plate, and the amount of receptor/*hedgehog* complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the *hedgehog* polypeptide, or which are reactive with the receptor protein and compete for binding with the *hedgehog* polypeptide; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the *hedgehog* polypeptide. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the *hedgehog* polypeptide. To illustrate, the *hedgehog* polypeptide can be chemically cross-linked or genetically fused with alkaline phosphatase, and the amount of *hedgehog* polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. paranitrophenylphosphate. Likewise, a fusion protein comprising the *hedgehog* polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) *J Biol Chem* 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as the anti-*hedgehog* antibodies described herein, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the *hedgehog* polypeptide



or *hedgehog* receptor sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ).

Where the desired portion of the *hedgehog* receptor (or other *hedgehog* binding molecule) cannot be provided in soluble form, liposomal vesicles can be used to provide manipulatable and isolatable sources of the receptor. For example, both authentic and recombinant forms of the *patched* protein can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374).

In addition to cell-free assays, such as described above, the readily available source of *hedgehog* proteins provided by the art also facilitates the generation of cell-based assays for identifying small molecule agonists/antagonists and the like. Analogous to the cell-based assays described above for screening combinatorial libraries, cells which are sensitive to *hedgehog* induction, e.g. *patched*-expressing cells or other epithelially-derived cells sensitive to *hedgehog* induction, can be contacted with a *hedgehog* protein and a test agent of interest, with the assay scoring for anything from simple binding to the cell to modulation in *hedgehog* inductive responses by the target cell in the presence and absence of the test agent. As with the cell-free assays, agents which produce a statistically significant change in *hedgehog* activities (either inhibition or potentiation) can be identified.

In other emdodiments, the cell-based assay scores for agents which disrupt association of *patched* and *smoothened* proteins, e.g., in the cell surface membrane or liposomal preparation.

In addition to characterizing cells that naturally express the *patched* protein, cells which have been genetically engineered to ectopically express *patched* can be utilized for drug screening assays. As an example, cells which either express low levels or lack expression of the *patched* protein, e.g. *Xenopus laevis* oocytes, COS cells or yeast cells, can be genetically modified using standard techniques to ectopically express the *patched* protein. (see Marigo et al., *supra*).

The resulting recombinant cells, e.g., which express a functional *patched* receptor, can be utilized in receptor binding assays to identify agonist or anatagonsts of *hedgehog* binding. Binding assays can be performed using whole cells. Furthermore, the recombinant cells of the

present invention can be engineered to include other heterologous genes encoding proteins involved in *hedgehog*-dependent signal pathways. For example, the gene products of one or more of *smoothed*, *costal-2* and/or *fused* can be co-expressed with *patched* in the reagent cell, with assays being sensitive to the functional reconstitution of the *hedgehog* signal transduction cascade.

Alternatively, liposomal preparations using reconstituted *patched* protein can be utilized. *Patched* protein purified from detergent extracts from both authentic and recombinant origins can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374). The lamellar structure and size of the resulting liposomes can be characterized using electron microscopy. External orientation of the *patched* protein in the reconstituted membranes can be demonstrated, for example, by immunoelectron microscopy. The *hedgehog* protein binding activity of liposomes containing *patched* and liposomes without the protein in the presence of candidate agents can be compared in order to identify potential modulators of the *hedgehog-patched* interaction.

The *hedgehog* protein used in these cell-based assays can be provided as a purified source (natural or recombinant in origin), or in the form of cells/tissue which express the protein and which are co-cultured with the target cells. As in the cell-free assays, where simple binding (rather than induction) is the *hedgehog* activity scored for in the assay, the protein can be labelled by any of the above-mentioned techniques, e.g., fluorescently, enzymatically or radioactively, or detected by immunoassay.

In addition to binding studies, functional assays can be used to identify modulators, i.e., agonists or antagonists, of *hedgehog* or *patched* activities. By detecting changes in intracellular signals, such as alterations in second messengers or gene expression, in *patched*-expressing cells contacted with a test agent, candidate agonists and antagonists to *patched* signaling can be identified.

A number of gene products have been implicated in *patched*-mediated signal transduction, including *patched*, the transcription factor *cubitus interruptus* (*ci*), the serine/threonine kinase *fused* (*fu*) and the gene products of *costal-2*, *smoothed* and *suppressor of fused*.

The interaction of a *hedgehog* protein with *patched* sets in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or translation of a gene. Potential transcriptional targets of *patched* signaling are the *patched* gene itself (Hidalgo and Ingham,

1990 *Development* 110, 291-301; Marigo et al., 1996 ) and the vertebrate homologs of the drosophila cubitus interruptus gene, the *GLI* genes (Hui et al. (1994) *Dev Biol* 162:402-413). *Patched* gene expression has been shown to be induced in cells of the limb bud and the neural plate that are responsive to *Shh*. (Marigo et al. (1996) *PNAS*, in press; Marigo et al. (1996) *Development* 122:1225-1233). The *GLI* genes encode putative transcription factors having zinc finger DNA binding domains (Orenic et al. (1990) *Genes & Dev* 4:1053-1067; Kinzler et al. (1990) *Mol Cell Biol* 10:634-642). Transcription of the *GLI* gene has been reported to be upregulated in response to *hedgehog* in limb buds, while transcription of the *GLI3* gene is downregulated in response to *hedgehog* induction (Marigo et al. (1996) *Development* 122:1225-1233). By selecting transcriptional regulatory sequences from such target genes, e.g. from *patched* or *GLI* genes, that are responsible for the up- or down regulation of these genes in response to *patched* signalling, and operatively linking such promoters to a reporter gene, one can derive a transcription based assay which is sensitive to the ability of a specific test compound to modify *patched* signalling pathways. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as agonists or antagonists of *ptc* induction of differentiation/quiescence.

Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on *ptc* signaling. To identify potential regulatory elements responsive to *ptc* signaling present in the transcriptional regulatory sequence of a target gene, nested deletions of genomic clones of the target gene can be constructed using standard techniques. See, for example, Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989); U.S. Patent 5,266,488; Sato et al. (1995) *J Biol Chem* 270:10314-10322; and Kube et al. (1995) *Cytokine* 7:1-7. A nested set of DNA fragments from the gene's 5'-flanking region are placed upstream of a reporter gene, such as the luciferase gene, and assayed for their ability to direct reporter gene expression in *patched* expressing cells. Host cells transiently transfected with reporter gene constructs can be scored for the induction of expression of the reporter gene in the presence and absence of *hedgehog* to determine regulatory sequences which are responsive to *patched*-dependent signalling.

In practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on second messengers generated by induction with *hedgehog* protein. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the *hedgehog* activity, with the level of expression of the reporter gene providing the *hedgehog*-

dependent detection signal. The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNase protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound (or *hedgehog*) or it may be compared with the amount of transcription in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the signal transduction of the *patched* protein, e.g., the test compound is a potential ptc therapeutic.

As described in further detail below, in preferred embodiments the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence. In other preferred embodiments, the reporter or marker gene provides a selective growth advantage, e.g., the reporter gene may enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug.

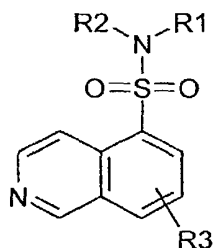
Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368).

Transcriptional control elements which may be included in a reporter gene construct include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is induced after modulation of a *patched* signal transduction pathway. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular stimulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription

requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

In yet other embodiments, second messenger generation can be measured directly in the detection step, such as mobilization of intracellular calcium, phospholipid metabolism or adenylyl cyclase activity are quantitated, for instance, the products of phospholipid hydrolysis IP<sub>3</sub>, DAG or cAMP could be measured. For example, recent studies have implicated protein kinase A (PKA) as a possible component of *hedgehog/patched* signaling (Hammerschmidt et al. (1996) *Genes & Dev* 10:647). High PKA activity has been shown to antagonize *hedgehog* signaling in these systems. Although it is unclear whether PKA acts directly downstream or in parallel with *hedgehog* signaling, it is possible that *hedgehog* signalling occurs via inhibition of PKA activity. Thus, detection of PKA activity provides a potential readout for the instant assays.

In a preferred embodiment, the *ptc* therapeutic is a PKA inhibitor. A variety of PKA inhibitors are known in the art, including both peptidyl and organic compounds. For instance, the *ptc* therapeutic can be a 5-isoquinolinesulfonamide, such as represented in the general formula:



wherein,

R<sub>1</sub> and R<sub>2</sub> each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, -(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>, -(CH<sub>2</sub>)<sub>m</sub>-OH, -(CH<sub>2</sub>)<sub>m</sub>-O-lower alkyl, -(CH<sub>2</sub>)<sub>m</sub>-O-lower alkenyl, -(CH<sub>2</sub>)<sub>n</sub>-O-(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>, -(CH<sub>2</sub>)<sub>m</sub>-SH, -(CH<sub>2</sub>)<sub>m</sub>-S-lower alkyl, -(CH<sub>2</sub>)<sub>m</sub>-S-lower alkenyl, -(CH<sub>2</sub>)<sub>n</sub>-S-(CH<sub>2</sub>)<sub>m</sub>-R<sub>8</sub>, or

R<sub>1</sub> and R<sub>2</sub> taken together with N form a heterocycle (substituted or unsubstituted);

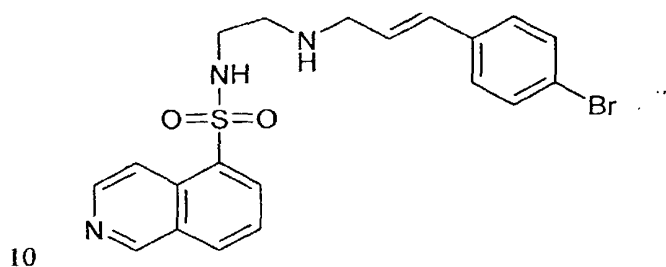
R<sub>3</sub> is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate,

or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido,  $-(CH_2)_m-R_8$ ,  $-(CH_2)_m-OH$ ,  $-(CH_2)_m-O$ -lower alkyl,  $-(CH_2)_m-O$ -lower alkenyl,  $-(CH_2)_n-O-(CH_2)_m-R_8$ ,  $-(CH_2)_m-SH$ ,  $-(CH_2)_m-S$ -lower alkyl,  $-(CH_2)_m-S$ -lower alkenyl,  $-(CH_2)_n-S-(CH_2)_m-R_8$ ;

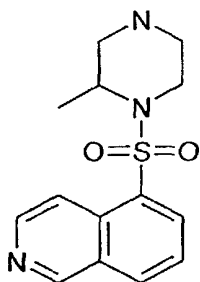
- 5  $R_8$  represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

$n$  and  $m$  are independently for each occurrence zero or an integer in the range of 1 to 6.

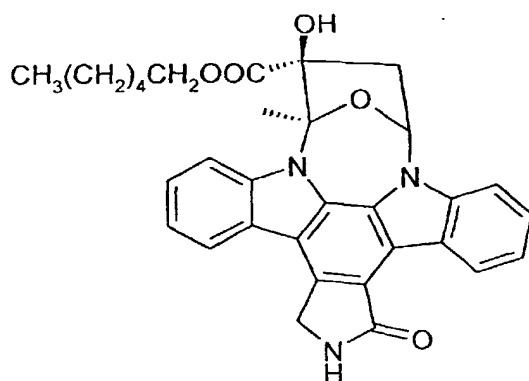
In a preferred embodiment, the PKA inhibitor is N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89; Calbiochem Cat. No. 371963), e.g., having the formula:



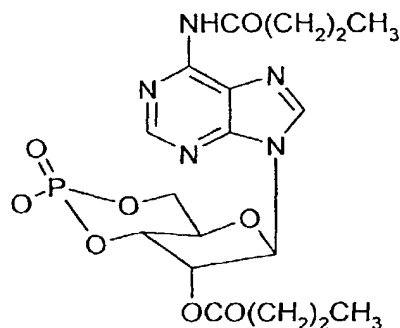
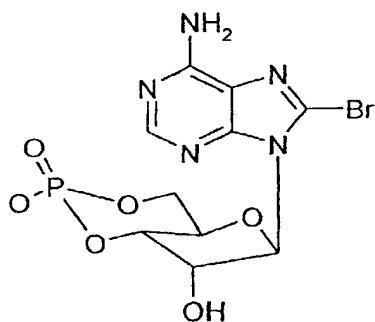
In another embodiment, the PKA inhibitor is 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7; Calbiochem Cat. No. 371955), e.g., having the formula:



- 15 In still other embodiments, the PKA inhibitor is KT5720 (Calbiochem Cat. No. 420315), having the structure



A variety of nucleoside analogs are also useful as PKA inhibitors. For example, the subject method can be carried out cyclic AMP analogs which inhibit the kinase activity of PKA, as for example, 8-bromo-cAMP or dibutyryl-cAMP



Exemplary peptidyl inhibitors of PKA activity include the PKA Heat Stable Inhibitor (isoform  $\alpha$ ; see, for example, Calbiochem Cat. No. 539488, and Wen et al. (1995) *J Biol Chem* 270:2041).

Certain *hedgehog* receptors may stimulate the activity of phospholipases. Inositol lipids can be extracted and analyzed using standard lipid extraction techniques. Water soluble derivatives of all three inositol lipids (IP<sub>1</sub>, IP<sub>2</sub>, IP<sub>3</sub>) can also be quantitated using radiolabelling techniques or HPLC.

The mobilization of intracellular calcium or the influx of calcium from outside the cell may be a response to *hedgehog* stimulation or lack thereof. Calcium flux in the reagent cell can be measured using standard techniques. The choice of the appropriate calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca<sup>++</sup>-sensitive microelectrodes depends on the cell type and the magnitude and time constant of the event under study (Borle (1990) *Environ Health Perspect* 84:45-56). As an exemplary method of Ca<sup>++</sup> detection, cells could be loaded

with the  $\text{Ca}^{++}$  sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in  $\text{Ca}^{++}$  measured using a fluorometer.

In certain embodiments of the assay, it may be desirable to screen for changes in cellular phosphorylation. As an example, the drosophila gene *fused* (*fu*) which encodes a serine/threonine kinase has been identified as a potential downstream target in *hedgehog* signaling. (Preat et al., 1990 *Nature* 347, 87-89; Therond et al. 1993, *Mech. Dev.* 44. 65-80). The ability of compounds to modulate serine/threonine kinase activation could be screened using colony immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430) using antibodies against phosphorylated serine or threonine residues. Reagents for performing such assays are commercially available, for example, phosphoserine and phosphothreonine specific antibodies which measure increases in phosphorylation of those residues can be purchased from commercial sources.

In yet another embodiment, the *ptc* therapeutic is an antisense molecule which inhibits expression of a protein involved in a *patched*-mediated signal transduction pathway. To illustrate, by inhibiting the expression of a protein which are involved in *patched* signals, such as *fused*, *costal-2*, *smoothened* and/or *Gli* genes, the ability of the *patched* signal pathway(s) to inhibit proliferation of a cell can be altered, e.g., potentiated or repressed.

As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions with cellular mRNA and/or genomic DNA encoding a *hedgehog* protein, *patched*, or a protein involved in *patched*-mediated signal transduction. The hybridization should inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the target cellular mRNA. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a target gene. Such oligonucleotide probes are preferably modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents



5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

Several considerations should be taken into account when constructing antisense oligonucleotides for the use in the methods of the invention: (1) oligos should have a GC content of 50% or more; (2) avoid sequences with stretches of 3 or more G's; and (3) oligonucleotides should not be longer than 25-26 mers. When testing an antisense oligonucleotide, a mismatched control can be constructed. The controls can be generated by reversing the sequence order of the corresponding antisense oligonucleotide in order to conserve the same ratio of bases.

In an illustrative embodiment, the *ptc* therapeutic can be an antisense construct for inhibiting the expression of *patched*, e.g., to mimic the inhibition of *patched* by *hedgehog*. Exemplary antisense constructs include:

5'-GTCCTGGCGCCGCCGCCGCCGTCGCC  
5'-TTCCGATGACCGGCCTTTCGCGGTGA  
5'-GTGCACGGAAAGGTGCAGGCCACACT

#### VI. Exemplary pharmaceutical preparations of *hedgehog* and *ptc* therapeutics

The source of the *hedgehog* and *ptc* therapeutics to be formulated will depend on the particular form of the agent. Small organic molecules and peptidyl fragments can be chemically synthesized and provided in a pure form suitable for pharmaceutical/cosmetic usage. Products of natural extracts can be purified according to techniques known in the art. For example, the Cox et al. U.S. Patent 5,286,654 describes a method for purifying naturally occurring forms of a secreted protein and can be adapted for purification of *hedgehog* polypeptides. Recombinant sources of *hedgehog* polypeptides are also available. For example, the gene encoding *hedgehog* polypeptides, are known, *inter alia*, from PCT publications WO 95/18856 and WO 96/17924.

Those of skill in treating epithelial tissues can determine the effective amount of an *hedgehog* or *ptc* therapeutic to be formulated in a pharmaceutical or cosmetic preparation.

The *hedgehog* or *ptc* therapeutic formulations used in the method of the invention are most preferably applied in the form of appropriate compositions. As appropriate compositions there may be cited all compositions usually employed for systemically or topically administering drugs. The pharmaceutically acceptable carrier should be substantially inert, so as not to act with

the active component. Suitable inert carriers include water, alcohol polyethylene glycol, mineral oil or petroleum gel, propylene glycol and the like.

To prepare the pharmaceutical compositions of this invention, an effective amount of the particular hedgehog or ptc therapeutic as the active ingredient is combined in intimate admixture with a pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical compositions are desirable in unitary dosage form suitable, particularly, for administration orally, rectally, percutaneously, or by parenteral injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in the case of powders, pills, capsules, and tablets. Because of their ease in administration, tablets and capsules represents the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations. In the compositions suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a suitable wetting agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not introduce a significant deleterious effect on the skin.

In addition to the direct topical application of the preparations they can be topically administered by other methods, for example, encapsulated in a temperature and/or pressure sensitive matrix or in film or solid carrier which is soluble in body fluids and the like for subsequent release, preferably sustained-release of the active component.

As appropriate compositions for topical application there may be cited all compositions usually employed for topically administering therapeutics, e.g., creams, gellies, dressings, shampoos, tinctures, pastes, ointments, salves, powders, liquid or semiliquid formulation and the like. Application of said compositions may be by aerosol e.g. with a propellant such as nitrogen carbon dioxide, a freon, or without a propellant such as a pump spray, drops, lotions, or a semisolid such as a thickened composition which can be applied by a swab. In particular

compositions, semisolid compositions such as salves, creams, pastes, gellies, ointments and the like will conveniently be used.

It is especially advantageous to formulate the subject compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discrete units suitable as unitary dosages, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powders packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The pharmaceutical preparations of the present invention can be used, as stated above, for the many applications which can be considered cosmetic uses. Cosmetic compositions known in the art, preferably hypoallergic and pH controlled are especially preferred, and include toilet waters, packs, lotions, skin milks or milky lotions. The preparations contain, besides the hedgehog or ptc therapeutic, components usually employed in such preparations. Examples of such components are oils, fats, waxes, surfactants, humectants, thickening agents, antioxidants, viscosity stabilizers, chelating agents, buffers, preservatives, perfumes, dyestuffs, lower alkanols, and the like. If desired, further ingredients may be incorporated in the compositions, e.g. antiinflammatory agents, antibacterials, antifungals, disinfectants, vitamins, sunscreens, antibiotics, or other anti-acne agents.

Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate. As examples of surfactants there may be cited anionic surfactants such as sodium stearate, sodium cetylsulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrochloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g. the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin. Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of lower alcohols include ethanol and isopropanol; examples of thickening agents

include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin; examples of chelating agents include disodium edetate and ethanedihydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

For preparing ointments, creams, toilet waters, skin milks, and the like, typically from 0.01 to 10% in particular from 0.1 to 5% and more in particular from 0.2 to 2.5% of the active ingredient, e.g., of the hedgehog or ptc therapeutic, will be incorporated in the compositions. In ointments or creams, the carrier for example consists of 1 to 20%, in particular 5 to 15% of a humectant, 0.1 to 10% in particular from 0.5 to 5% of a thickener and water; or said carrier may consist of 70 to 99%, in particular 20 to 95% of a surfactant, and 0 to 20%, in particular 2.5 to 15% of a fat; or 80 to 99.9% in particular 90 to 99% of a thickener; or 5 to 15% of a surfactant, 2-15% of a humectant, 0 to 80% of an oil, very small ( $< 2\%$ ) amounts of preservative, coloring agent and/or perfume, and water. In a toilet water, the carrier for example consists of 2 to 10% of a lower alcohol, 0.1 to 10% or in particular 0.5 to 1% of a surfactant, 1 to 20%, in particular 3 to 7% of a humectant, 0 to 5% of a buffer, water and small amounts ( $< 2\%$ ) of preservative, dyestuff and/or perfume. In a skin milk, the carrier typically consists of 10-50% of oil, 1 to 10% of surfactant, 50-80% of water and 0 to 3% of preservative and/or perfume. In the aforementioned preparations, all % symbols refer to weight by weight percentage.

Particular compositions for use in the method of the present invention are those wherein the hedgehog or ptc therapeutic is formulated in liposome-containing compositions. Liposomes are artificial vesicles formed by amphiphatic molecules such as polar lipids, for example, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebiosides. Liposomes are formed when suitable amphiphatic molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material (also referred to as coarse liposomes). Another type of liposome known to be consisting of a single bilayer encapsulating aqueous material is referred to as a unilamellar vesicle. If water-soluble materials are included in the aqueous phase during the swelling of the lipids they become entrapped in the aqueous layer between the lipid bilayers.

Water-soluble active ingredients such as, for example, various salt forms of a hedgehog polypeptide, are encapsulated in the aqueous spaces between the molecular layers. The lipid soluble active ingredient of hedgehog or ptc therapeutic, such as an organic mimetic, is

predominantly incorporated into the lipid layers, although polar head groups may protude from the layer into the aqueous space. The encapsulation of these compounds can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation from an organic solvent. When this film is dispersed in a suitable aqueous medium, multilamellar liposomes are formed. Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble active ingredients are usually incorporated by dispersing the cast film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysis or other art-known suitable procedures. The lipid-soluble active ingredient is usually incorporated by dissolving it in the organic solvent with the phospholipid prior to casting the film. If the solubility of the material in the lipid phase is not exceeded or the amount present is not in excess of that which can be bound to the lipid, liposomes prepared by the above method usually contain most of the material bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required.

A particularly convenient method for preparing liposome formulated forms of hedgehog and ptc therapeutics is the method described in EP-A-253,619, incorporated herein by reference. In this method, single bilayered liposomes containing encapsulated active ingredients are prepared by dissolving the lipid component in an organic medium, injecting the organic solution of the lipid component under pressure into an aqueous component while simultaneously mixing the organic and aqueous components with a high speed homogenizer or mixing means, whereupon the liposomes are formed spontaneously.

The single bilayered liposomes containing the encapsulated hedgehog or ptc therapeutic can be employed directly or they can be employed in a suitable pharmaceutically acceptable carrier for topical administration. The viscosity of the liposomes can be increased by the addition of one or more suitable thickening agents such as, for example xanthan gum, hydroxypropyl cellulose, hydroxypropyl methylcellulose and mixtures thereof. The aqueous component may consist of water alone or it may contain electrolytes, buffered systems and other ingredients, such as, for example, preservatives. Suitable electrolytes which can be employed include metal salts such as alkali metal and alkaline earth metal salts. The preferred metal salts are calcium chloride, sodium chloride and potassium chloride. The concentration of the electrolyte may vary from zero to 260 mM, preferably from 5 mM to 160 mM. The aqueous component is placed in a suitable vessel which can be adapted to effect homogenization by effecting great turbulence during the injection of the organic component. Homogenization of the two components can be accomplished within the vessel, or, alternatively, the aqueous and organic components may be injected separately into a mixing means which is located outside the vessel. In the latter case, the

liposomes are formed in the mixing means and then transferred to another vessel for collection purpose.

5 The organic component consists of a suitable non-toxic, pharmaceutically acceptable solvent such as, for example ethanol, glycerol, propylene glycol and polyethylene glycol, and a suitable phospholipid which is soluble in the solvent. Suitable phospholipids which can be employed include lecithin, phosphatidylcholine, phosphatidylserine, phosphatidylethanol-amine, phosphatidylinositol, lysophosphatidylcholine and phosphatidyl glycerol, for example. Other lipophilic additives may be employed in order to selectively modify the characteristics of the liposomes. Examples of such other additives include stearylamine, phosphatidic acid, 10 tocopherol, cholesterol and lanolin extracts.

In addition, other ingredients which can prevent oxidation of the phospholipids may be added to the organic component. Examples of such other ingredients include tocopherol, butylated hydroxyanisole, butylated hydroxytoluene, ascorbyl palmitate and ascorbyl oleate. Preservatives such as benzoic acid, methyl paraben and propyl paraben may also be added.

15 Apart from the above-described compositions, use may be made of covers, e.g. plasters, bandages, dressings, gauze pads and the like, containing an appropriate amount of a hedgehog or ptc therapeutic. In some cases use may be made of plasters, bandages, dressings, gauze pads and the like which have been impregnated with a topical formulation containing the therapeutic formulation.

20

### *Exemplification*

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the 25 invention.

#### *Purification of hedgehog protein.*

Human sonic hedgehog protein (residues 24-197) was expressed in the baculovirus/insect cell system (Roelink et al. (1995) *Cell* 81:445-455). The conditioned medium was loaded onto 30 Fast Flo SP agarose equilibrated with 50 mM potassium phosphate, 0.5 mM DTT, pH 7.0. The column was washed with this buffer, and then eluted with a gradient to 10. M NaCl. Fractions were assayed for the induction of alkaline phosphatase activity on mesenchymal stem cells (C3H10T1/2 cells, see, e.g., Wang et al. (1993) *Growth Factors* 9:57-71) and then pooled on the

basis of this activity and also by purity on SDS gels. The pooled material was concentrated on an Amicon ultra filtration unit (PM10 membrane) and diafiltered against 10 mM Tris, pH 7.4, 0.5 mM DTT. Protein was estimated by the Bradford method using gamma globulin as a standard.

5 *Preparation of collagen sponge.*

Collagen sponge was washed extensively in MilliQ water to remove any surfactants and additives from the manufacturer. The sponge was then washed in 70% ethanol, then dried in vacuo.

10 *Preparation of implants.*

Protein was added to 1.0 - 1.5 mm by 8-10 mm pieces of collagen sponge (1.5-3.0 mg in weight). In some cases zinc sulfate was added to a final concentration of 0.2 mM before the hedgehog protein was added to the collagen sponge. The reconstituted sponges were then frozen and lyophilized.

15

*Implantation.*

20

Sponges were implanted either subcutaneously in the thoracic region of Sprague Dawley rats (4-8 weeks old) or in the thigh muscle of rabbits (11-14 weeks old). Animals were maintained for 2-5 weeks before removing the implant. The implant was then fixed in 4% formalin or 4% paraformaldehyde and then embedded in JB-4 resin. Sections were stained with toluidine blue (Wang et al. (1988) *PNAS* 85:9484-9488).

25

The induction of new hair follicles, sebaceous glands, and other dermal structures were identified by its distinctive morphology. The careful subcutaneous or intramuscular placement of our implants and the careful removal of these implants preclude the possibility of contamination from existing dermal structures. Also, the appearance of more immature hair follicles is seen in the implants of shorter (2 week) duration.

30

Biopsy slides were obtained from an intramuscular implant taken out of a rabbit muscle at three weeks and stained with hematoxylin and eosin. Similar slides were examined of an intramuscular implant, rabbit muscle, three weeks, stained with toluidine blue. Slides of certain samples revealed a tissue morphology indicating the presence of follicle- and hair-like structures forming in the intramuscular tissue.

*Hair induction by Shh*

- As a follow-up to the above experiments, hedgehog-loaded collagen sponges were implanted under the shaved skin of mice. As indicated in Figures 1A-C, the hedgehog preparations were able to induce hair growth over the implants. Moreover, Ihh protein modified at the C terminus with a Von Willebrand's factor collagen binding site was active in hair growth, indicating a localized inducing activity of the implanted protein.
- 5

All of the above-cited references and publications are hereby incorporated by reference.

*Equivalents*

- Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.
- 10

0937410-040504  
FOUO-072860